Please replace the first full paragraph on page 34 with the following:

Figure 11 shows the results obtained from emission spectral analysis. The spectra have been normalized to allow easier visualization of the emission wavelength data. Figure 9 shows the reversibility of denaturation for an entrapped protein.

IN THE CLAIMS:

In accordance with 37 C.F.R. § 1.121, please substitute for claim 55 the following rewritten version of the same claim, as amended. The changes are shown explicitly in the attached "Marked-Up Version of Amended Claims to Show Changes Made."

50 <- 55. (Amended) A carrier comprising a matrix of inorganic, organic, or organic and inorganic material and containing a biomolecular interaction entrapped within the matrix wherein the biomolecular interaction comprises two or more biological species that can be reversibly dissociated from the other under dissociating conditions.

REMARKS

The Official Action dated October 22, 2002 has been carefully considered. It is believed that the following comments represent a complete response to the Examiner's rejections and place the present application in condition for allowance. Reconsideration is respectfully requested.

I. Summary of the Amendments to the Specification and Claims

The application has been amended to correct minor typographical errors. In addition, 50 claim 55 has been amended to recite that the biomolecular interaction comprises two or more biological species that can be reversibly dissociated from the other "under dissociating conditions." *

It is acknowledged that the foregoing amendments are submitted after final rejection. However, because the amendments either place the application in condition for allowance, or at least in better condition for appeal, entry thereof by the Examiner is respectfully requested.

II. Rejection of the Claims Under 35 USC § 112, First Paragraph

Claims 50 - 65 were rejected under 35 U.S.C. § 112, first paragraph, on the basis that the claims were not enabled by the disclosure. Office Action at page 2. In support of this ground for rejection, the Examiner stated that the disclosure does not teach a person skilled in the art to practice the claimed invention without undue experimentation. The Examiner further stated that the application only teaches a person skilled in the art how to dissociate a biomolecular interaction in the presence of urea, GdHCl, or under thermal conditions. Applicants traverse this rejection as follows.

First, it should be noted that the present application is not directed to "novel" biomolecular interactions *per se*. The claims currently under consideration are directed to a carrier that comprises a biomolecular interaction entrapped within a matrix, wherein the biomolecular interaction comprises at least two biological species that can be reversibly dissociated from the other within the matrix. One of the main embodiments of the invention is a composition of matter that can be used to study what affects the dissociation or association of biological species within a biomolecular interaction. The ability to reversibly dissociate within the matrix is important and is a characteristic that defines the carrier claimed; however, the claims do not need to be limited to specific dissociating factors. This is especially true as one of the embodiments of the invention is to identify and study dissociating factors and a person skilled in the art would know that dissociating factors may vary depending on the matrix used, the biomolecular interaction, and the use of the carrier. For instance, there are many prior art references on the disruption of protein-protein interactions, such as:

1. Orosz et al., "Effects of calmodulin antagonists on antibody binding to calmodulin. Distinct conformers of calmodulin induced by the binding of drugs," *Biochemical Journal, 284*(3):803-8 (1992);

2. Erickson et al., "Reverse two-hybrid method for screening for molecules inhibiting protein-protein interactions," PCT Int. Appl. No. WO 95/26400 (1995);

3. Fields et al., "The two-hybrid system: an assay for protein-protein interactions," *Trends in Genetics*, *10*(8):286-92 (1994); and

4. Fernandes et al., "Technological advances in high-throughput screening," *Current Opinion in Chemical Biology*, *2*(5):597-603 (1998).

A person skilled in the art upon reading the description of the present application would appreciate that the reversible dissociation in the invention can be caused by denaturants or other molecules, such as antagonists of the biomolecular interaction. It could also be that the biological species of the biomolecular interaction are coupled and then dissociated upon the addition of an antagonist or a change in other conditions such as pH, temperature, GdHCl, or urea levels. In one embodiment, the claimed invention provides a screening method for potential antagonists and dissociating factors. Even if one does not specify the antagonist or denaturant factor, the matrix and biomolecular interaction will be such that it can reversibly dissociate within the matrix.

The biomolecular interaction can start in a complexed configuration and the dissociation can be monitored in the presence of potential antagonists. Alternatively, the biomolecular interaction can be in a non-complexed form and the formation of the complex can be monitored in the presence of potential antagonists or other conditions. This is described in the application at page 7, lines 3 – 9, wherein the initial florescence signal of the biomolecular interaction in the complex is first measured and then subsequently the complex is reversibly disrupted.

Although denaturing the complex is one embodiment of the invention and is useful especially when the biomolecular interaction complex is tightly associated or has slow on/off rates, the invention should not be so limited. Some inhibitors or dissociating factors can act allosterically, *i.e.*, by binding to a site away from the interface that induces a conformational change in a protein and dissociation of the interaction. Alternatively, the complex can be dissociated directly by antagonists if the on/off rates for formation of the complex are such that there is sufficient dynamic motion to allow a small molecule to insert into the interfacial region, thus dissociating the protein complex. These concepts were known in the art. As such a person skilled in the art would be aware that the complex could be denatured or dissociated by other means, such as a compound that disrupts the complex, as suggested at page 2, line 1, of the application, and the invention would not be limited by the use of specific denaturants *per se* or by other environmental or matrix conditions.

This was also confirmed in a subsequent paper by the inventors enclosed herewith (*Analytca Chimica Acta, 470*:19-28 (2002). See 3.3, pages 24-26 and "Conclusions" at page 27, where it is stated that a protein-peptide interaction in a sol-gel derived material can be dissociated by antagonists). At column 1, end of the first paragraph, the paper states that "[T]he ability of small molecule inhibitors to enter the glass and reversibly dissociate the complex indicated that the entrapped complex is suitable for screening of antagonists in a high-throughput format".

As such, it should not be necessary to specifically set out and have examples for each and every potential combination of components that can be used to dissociate biological species in a particular biomolecular interaction. What the inventors need to show is that a biomolecular interaction can be incorporated into a matrix in a manner wherein it is entrapped but can be reversibly dissociated. The claimed invention enables a person skilled in the art to do just that. The present inventors have shown that one way to study, use, and manipulate biomolecular interactions is to entrap the components in a matrix that enables the interaction to be reversibly dissociated and thus enabling one to manipulate, use, and/or further study the interaction. The conditions under which this is done would not require undue experimentation by a person skilled in the art especially as the best mode of practicing the invention and scientific principles of the invention are clearly described.

III. Conclusion

In view of the foregoing, it is submitted that the application is in condition for allowance and an early indication to that effect would be greatly appreciated.

The Examiner is invited to contact the undersigned by telephone if it is felt that an interview would advance the prosecution of the present application.

If there are any fees due in connection with the filing of this Amendment, please charge the fees to our Deposit Account No. 19-0741. If a fee is required for an extension of time under 37 C.F.R. § 1.136 not accounted for above, such an extension is requested and the fee should also be charged to our Deposit Account.

Respectfully submitted,

Date Jan 22, 2003

By Michele M Simkin

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Attorney for Applicant
Registration No. 34,717

Marked Up Version of the Changes to the Specification and Claims

IN THE SPECIFICATION:

Please replace the last full paragraph on page 2 with the following:

According to another embodiment the present invention provides a carrier containing a biomolecular interaction wherein the biomolecular **[bimolecular]** interaction is bioactive. According to another embodiment the carrier is also bioactive, preferably either bioactive form is pre-treated to contain components found in an animal fluid, preferably the pre-treatment is by immersion in a solution containing components found in an animal fluid for a period of up to about seven days prior to use. According to one embodiment of this aspect of the invention the animal fluid is interstitial fluid. Preferably such carrier is synthesized under sterile conditions or sterilized subsequent to synthesis using conventional sterilization methods.

Please replace the first full paragraph on page 3 with the following:

In another embodiment of this bioactive aspect of the biomolecular **[bimolecular]** interaction, where the carrier may also be bioactive, the carrier provides controlled release of the biologically active biomolecular interaction over time.

Please replace the third full paragraph on page 3 with the following:

In another aspect of the invention there are provided methods of preparing the carrier and the biomolecular **[bimolecular]** interaction incorporated in the carrier.

Please replace the third full paragraph on page 4 with the following:

According to yet another embodiment **[emboiment]** the partial drying is at temperatures from about 4° to about 40°C.

Please replace the fourth full paragraph on page 4 with the following:

In another aspect of the invention there is provided a method for the preparation of a carrier having a bioactive biomolecular **[bimolecular]** interaction incorporated in the carrier.

Please replace the last full paragraph on page 4 with the following:

According to one embodiment of this aspect, the method comprises:

(a) incorporating the bioactive biomolecular interaction in a carrier;

(b) hydrolysis and polycondensation of at least one monomer to provide a solid matrix bonding the bioactive biomolecular interaction which is incorporated **[incorpoerated]** in the carrier ; and

(c) imparting mechanical, chemical and thermal stability in the matrix.

At page 5, please replace the fourth full paragraph with the following:

In yet another aspect of the invention there is provided a method of treating an animal with a carrier and a biomolecular **[bimolecular]** interaction of the invention. According to one embodiment the method comprises administering an effective amount of a biologically active biomolecular interaction contained in a carrier such that the animal is thereby treated. Preferably the treating is by site-specific targeting in the animal. More preferably the effective amount of a biologically active biomolecular interaction is a chemotherapeutic for treating cancer.

Please replace the paragraph bridging pages 6 and 7 with the following:

According to another broad aspect the invention provides materials and methods of high throughput screening for compounds or substances which inhibit protein-protein interactions, but as will readily be appreciated, the methods and materials described herein may be used to assay any substance-substance interactions. In this respect the invention provides a method of high through put screening for a substance which inhibits or binds a biomolecular **[bimolecular]** interaction. According to an embodiment of this aspect of the invention the method comprises the steps of:

(a) incorporating a biomolecular **[bimolecular]** interaction within a carrier. Preferably the biomolecular **[bimolecular]** interaction is a population of a purified protein comprising a binary protein-protein interacting pair is encapsulated in a carrier. This protein is preferably designed so that it has a solvent-sensitive fluorescent probe at or near the dimer interface or has one protein partner labelled with a fluorescence energy donor and the other protein partner labelled with a fluorescence energy acceptor to allow fluorescence resonance

energy transfer (FRET) to occur between the protein partners, preferably in a distance-dependent manner.

(b) forming an array of sol-gel derived spots on a support wherein each spot contains a biomolecular **[bimolecular]** interaction;

(c) measuring a signal from the interaction in the absence of any other substances; Preferably the initial fluorescence signal (intensity, emission wavelength, polarization and/or lifetime of a single probe, a donor and/or an acceptor) of this complex in the absence of any other compounds is first measured. The fluorescence signal indicates that the pair of proteins is in a complex.

(d) reversibly disrupting the biomolecular interaction **[bimoleular interactin]** such that the signal is detectably **[detectabliy]** altered; Preferably the sample is sufficiently denatured (if necessary) to cause a reversible disruption of the binary protein-protein interaction, causing the signal of the reporter at the protein-interface or of the donor-acceptor pair to be significantly altered.

(e) the substance is added to the biomolecular **[bimolecular]** interaction in the carrier, and reversing the disruption; and

(f) measuring the signal; The denaturing conditions (if used) are reversed to cause the binary interacting system to reform in the sol-gel matrix. The original fluorescence signal of the attached probe or donor-acceptor pair is recovered if no disruption of the complex occurs (i.e., target is not a drug candidate). Where the original signal is not recovered, the substance is determined **[determned]** to bind or inhibit the biomolecular **[bimolecular]** interaction. The substance is considered to have disrupted the protein-protein interaction, and can be tested further as, for example, a potential drug candidate.

At page 7, please replace the first full paragraph with the following:

In a preferred **[prefered]** embodiment the signal generated by the assay is due to excitement by a He Cd laser through an optical fiber and the signal, preferably fluorescence, and is preferably detected through the same fiber, as shown in Figure 21.

At page 10, please replace the first full paragraph with the following:

As used herein, the expression "interfere with" and like expressions mean inhibiting, reducing, diminishing or blocking the interaction between the members of a biomolecular **[bimolecular]** interaction.

At page 10, please replace the second full paragraph with the following:

The term "interaction" as used herein means the binding, association, or complexing of the members of a biomolecular **[bimolecular]** interaction and includes intramolecular binding, association or complexing, regardless of the degree of association between molecules, or intra molecularly, however the association is formed.

At page 10, please replace the sixth full paragraph with the following:

The term "containing" as used herein means any form or type of integration, or incorporation such that the substance or substances, including biomolecular **[bimolecular]** interaction, is/are able to be carried by a carrier.

At page 22, please replace the second full paragraph with the following:

The invention contemplates a method for screening a compound to determine the degree of inhibition or stimulation (i.e. binding) of a biomolecular interaction by the compound, or if it interferes with the biomolecular **[bimolecular]** interaction. The method involves contacting a compound to be tested with molecules of a biomolecular interaction incorporated within a carrier as described herein. The molecules are preferably not complexed but are capable of forming a biomolecular interaction in the carrier. Therefore, a method of the invention may initially involve disrupting a biomolecular interaction by applying a denaturant (e.g. temperature, pH, or urea). The denaturation of the interaction may be achieved using a cyclic denaturation (temperature, urea, or pH cycling). The screening method is carried out under conditions suitable for the formation of a biomolecular interaction. The conditions are selected having regard to factors such as the nature of the molecules forming the interaction. In a preferred method of the invention the carrier is a silica based glass, most preferably a sol-gel derived glass. The biomolecular interaction within a carrier is preferably cast on the surface of an optical fiber, planar supports (waveguides), a slide, a microtiter well or an array of sol-gel derived spots formed by pin spotting, stamping, inkjet deposition or screen printing.

Please replace the first full paragraph on page 33 with the following:

Once it was determined that reversible denaturation of the entrapped complex was possible, we examined the ability of a known inhibitor of the complex, trifluoperazine (TFP), to disrupt the complex. Control experiments involved an examination of the interactions of TFP with the intact complex, melittin, BCaM, and non-functional complex, obtained using apo BCaM. Interaction of TFP with apo or holo calmodulin alone resulted in no changes in spectroscopic properties. The interaction of TFP with melittin and with the intact BCaM:melittin complex are shown in Figures 10A and 10B. Note that no wavelength **[wavlength]** changes occurred during this titration (not shown).[.]

Please replace the first full paragraph on page 34 with the following:

Figure 11 shows the results obtained from emission spectral analysis. The spectra have been normalized to allow easier visualization of the emission wavelength data. Figure 9 shows **[shows]** the reversibility of denaturation for an entrapped protein.

IN THE CLAIMS:

55. (Amended) A carrier comprising a matrix of inorganic, organic, or organic and inorganic material and containing a biomolecular interaction entrapped within the matrix wherein the biomolecular interaction comprises two or more biological species that can be reversibly dissociated from the other under dissociating conditions.

Biochem J (1992) 284, 803–808 (Printed in Great Britain)

Effects of calmodulin antagonists on antibody binding to calmodulin

Distinct conformers of calmodulin induced by the binding of drugs

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An indirect enzyme-linked immunosorbent assay has been used to study the interactions between calmodulin and two calmodulin antagonists, trifluoperazine and a neuropeptide isolated from the hypothalamus. The binding of a monospecific anti-calmodulin antibody, raised in rabbit against dinitrophenylated calmodulin, to calmodulin was tested at various concentrations of these drugs under equilibrium conditions. Trifluoperazine at low concentrations stimulated, but at relatively high concentrations inhibited, immunocomplex formation. The neuropeptide displaced the antibody from calmodulin at nanomolar concentrations. Enzyme-linked immunosorbent assays were also carried out with the large tryptic fragments of calmodulin. The results suggest that (i) the *C*-terminal fragment binds the antibody with an affinity which is comparable with that of intact calmodulin; (ii) the neuropeptide can form complexes with both *N*- and *C*-terminal fragments, but with two orders of magnitude less activity in case of the *C*-terminal fragment; and (iii) trifluoperazine does not stimulate antibody binding to the *C*-terminal fragment. Therefore the tertiary structure of calmodulin must be intact to ensure long-distance interactions between the binding sites of trifluoperazine, the neuropeptide and the antibody. These interactions may produce distinct conformers of calmodulin which may exhibit altered potency, not only for antibody binding but also for stimulation/inhibition of target enzymes.

INTRODUCTION

Calmodulin (CaM) is a ubiquitous Ca^{2+}-binding protein, the structure of which undergoes large Ca^{2+}-dependent changes [1]. The hydrophobic binding sites on its surface bind and activate/inhibit as many as 30 different proteins. (See refs. [2–5] for recent reviews).

Certain drugs can bind to these sites with high affinity and moderate the interactions of CaM. Detailed studies on the structural properties of CaM and some CaM antagonists have been published [6,7], but very little is still known about the molecular consequences of the interaction of CaM with compounds of anti-CaM activity. Since these compounds are profoundly different both structurally and pharmacologically, the finding that CaM interacts with all of them is indeed intriguing. It is also uncertain how anti-CaM drugs can bind to CaM and modify the stimulatory/inhibitory effects of CaM on different enzymes.

Trifluoperazine (TFP), a phenothiazine which is the classic CaM antagonist, binds to two distinct sites, one on the *C*-terminal and one on the *N*-terminal domain of CaM [8], and it antagonizes CaM-binding target enzymes. Five glycopeptides with coronaro-constrictory properties have been isolated from bovine hypothalamus [9], and determination of their amino acid compositions has shown high Ser, Gly and Glu content [9]. These peptides stimulate the CaM-sensitive activity of myosin light chain kinase [10] and influence the activity of CaM-dependent cyclic nucleotide phosphodiesterase [11]. We have shown that CaM is a potent target for these hypothalamic peptides [12]. The most effective CaM antagonist is investigated in the present study.

Drabikowski and his co-workers have shown that under controlled conditions in the presence of Ca^{2+}, CaM can be cleaved by trypsin into two large fragments at a well-defined cleavage site [13,14]. These large fragments of calmodulin obtained by limited trypsin digestion were used to study how the different target enzymes interact with the binding domain of CaM. It has been found that different fragments stimulate the CaM-dependent cyclic nucleotide phosphodiesterase [15] and the erythrocyte Ca^{2+}-ATPase [16]. Similar experiments were carried out with two amphipathic peptides, melittin [17] and mastoparan [18]. It has been shown that melittin can form complexes with both tryptic fragments, but with three orders of magnitude less affinity compared with binding to CaM itself [17]. Mastoparan has a binding affinity to the isolated *C*-terminal tryptic fragment of CaM that is almost the same as to intact CaM [18].

Antibodies can be used as highly specific reagents to study the binding of ligands as well as macromolecules to antigens. A crucial step in the formation of an immunocomplex is the specific recognition of the antigen by the antibody. The antigen–antibody reaction in a homogeneous liquid phase is a well-studied process (for review, see [19]). A sensitive and simple technique for investigation of the binding of antibody to CaM or CaM–drug complexes is a solid-phase method, such as e.l.i.s.a. In this method one of the reactants is immobilized on a solid surface.

We have developed an experimental system in which binding of the antibody to the solid-phase-bound calmodulin has been studied under equilibrium conditions [20,21]. Competitive e.l.i.s.a. experiments have shown that the affinity of antibody for calmodulin on the surface and in solution is nearly the same [21]. This finding suggests that antibody binding measured by e.l.i.s.a. may reflect the intrinsic affinity of the antibody measured in solution, and that the global conformational change of CaM due to immobilization can be excluded. In consequence, the e.l.i.s.a method can be used to study the effects of drugs on the accessibility of the antigenic site of CaM.

Abbreviations used: CaM, calmodulin; TFP, trifluoperazine.

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† To whom correspondence should be addressed.

In this study, we have investigated the interaction between calmodulin and two calmodulin antagonists of different chemical structure and biological activity using indirect e.l.i.s.a. The results of experiments with *N*- and *C*-terminal fragments of CaM argue for the importance of the native conformation of CaM in the binding processes. This approach renders it possible to differentiate among the drug-binding sites and to demonstrate co-operative interactions within the CaM molecule.

MATERIALS AND METHODS

TFP was kindly provided by Chinoin Chemical Works (Budapest, Hungary). Acetonitrile (LiChrosolv, gradient grade) was from Merck (Darmstadt, Germany). All other chemicals were high purity commercial preparations from Reanal (Budapest, Hungary).

A coronaro-constrictory peptide (molecular mass 3 kDa) was isolated from bovine hypothalamus by gel filtration on Sephadex G-10, ion-exchange chromatography on Dowex 50W × 8 and semipreparative and analytical reverse-phase h.p.l.c. using a LiChrosorb C-8 column (16 mm × 250 mm) and a Nucleosil C-18 column (4.6 mm × 250 mm) respectively [9].

CaM from bovine brain was purified to homogeneity using TFP–Sepharose-4B chromatography [22]. Rabbits were immunized with 1-fluoro-2,4-dinitrobenzene-derivatized bovine CaM as described in [23]. The IgG fraction was isolated from the antiserum using the QAE-Sephadex A-50 (Pharmacia) chromatographic method [24]. Monospecific antibodies were further purified on a CaM–Sepharose-4B affinity column [20,24], then concentrated in an Amicon ultrafiltration cell, dialysed against 0·05 M-sodium carbonate/bicarbonate buffer, pH 9.6, and stored at 4 °C in the presence of 0·02% NaN_3.

Standard e.l.i.s.a. protocols [25] for indirect assays were used with some modifications as follows. For the indirect non-competitive assay, coating of 96-well microtitre plate with CaM in the presence of 1 mM-Ca^{2+} was carried out for 18 h at 6 °C [12]. Antibodies were diluted in wells, and the plate was allowed to stand for 2 h at room temperature. Goat anti-(rabbit Ig) conjugated with horseradish peroxidase (Human, Budapest, Hungary) at 1·1000 dilution was allowed to interact for 2 h at room temperature. *O*-Phenylenediamine 3·7 mM, with peroxide added, was used as the substrate solution. Absorbances were read at 492 nm after 1 h of substrate hydrolysis.

To study the effect of drugs on immunocomplex formation, plates were coated with 2.5 μg of CaM/ml (in the presence of 1 mM-Ca^{2+}). Dilutions of CaM antagonists were prepared on a separate plate and antibodies were added to each well to give a final antibody concentration of 2.5 μg/ml. The plate was allowed to stand for 1 h at 4 °C. The total content of each well was then transferred to the appropriate wells of the coated plate and allowed to stand for 2 h at room temperature. This incubation time was sufficient to obtain equilibrium values. Subsequent steps were the same as those described for the non-competitive assay.

CaM was digested with trypsin as described in [13] in the presence of 0.1 mM-$CaCl_2$ in a solution of 2 mg of CaM/ml. The fragments were purified by using reverse-phase h.p.l.c. as described below. Both fragments were homogeneous when examined by electrophoresis on a 12.5% polyacrylamide gel in 8 M-urea.

CaM fragments were isolated on reverse-phase h.p.l.c. [16], using a LiChrosorb 5 RP-18 column (4.1 mm × 250 mm; 5 μm particle size; Bio Separation Technologies, Budapest, Hungary) and a Waters (Milford, MA, U.S.A.) U6K injector. Two Waters h.p.l.c. pumps (model 510) were regulated by a Waters automated gradient controller (model 680). The effluent was monitored at 214 nm using a Waters absorbance detector (model 441). The data were analysed by a Waters digital integrator (model 740). All solutions were purified on a 0.45 μm-pore-size filter (Micro Filtration Systems, Dublin, CA, U.S.A.) and degassed before use. Details of the gradient are given in the legend to Fig. 3. The fragments were collected, lyophilized and stored at −20 °C.

RESULTS

Optimization of indirect e.l.i.s.a.

An effective method for the study of the interaction of CaM with biologically active compounds is indirect e.l.i.s.a.; the immobilized CaM captures the antibody from the test sample depending on its reactivity, which is then detected by the enzyme-labelled anti-IgG. It has been reported that CaM has a single antigenic site [23,26] within the *C*-terminal domain [26,27], and that the monospecific antibody binds to a continuous segment of amino acid residues (137–143). To ascertain that this is indeed the case with the antibody used in our experiments, a set of 'sandwich' e.l.i.s.a. measurements was carried out. In this experiment at least two antibodies, enzyme-labelled and non-labelled, should have reacted with the same molecule of CaM. However, horseradish peroxidase-conjugated antibody could not react with the immobilized antibody–CaM complex [20].

Based on the optimization experiments [20], 2.5 μg of CaM/ml and 2.5 μg of antibody/ml were found to ensure the very sensitive detection of binding between antigen and antibody. Fig. 1 shows the data of antibody titration at a concentration of coated CaM of 2.5 μg/ml at equilibrium. After the antigen–antibody reaction the second immunoglobulin (anti-antibody) conjugate was added at a 1:1000 dilution, which provides a broad range in the linear portion of the e.l.i.s.a. plot and a defined plateau in the region of antigen saturation [21]. Therefore the A_{492} values can be used to determine the amount of antibody bound to the immobilized CaM.

Effect of TFP and the neuropeptide on the binding of antibody to CaM

In one set of experiments, TFP or the neuropeptide at various concentrations were added to the optimized indirect e.l.i.s.a. assay and A_{492} values were read. As shown in Fig. 2, the binding of antibody to CaM depends on both the chemical structure and the concentration of the molecules.

TFP, the classic CaM antagonist, affected immunocomplex formation in a complicated way. At relatively low drug concentrations it stimulated the binding of antibody to the intact CaM, with a maximum at 3.9 μM-TFP, whereas at higher concentrations TFP displaced or prevented the binding of antibody to CaM. It should be noted that the concentration of TFP at which maximum binding appears depends on the concentration and the reactivity of antibody used in a given experiment (results not shown).

A neuropeptide isolated from the hypothalamus [9] is a potent inhibitor of the formation of immunocomplex [12] (cf. Fig. 2). The binding of antibody to CaM was progressively inhibited by increasing the concentration of the peptide and the inhibition was already complete at very low peptide concentrations.

Interaction of tryptic calmodulin fragments with the anti-calmodulin antibody

To investigate the complex binding of TFP, e.l.i.s.a. experiments were carried out on a CaM tryptic digest mixture and on the purified *N*- and *C*-terminal fragments. The same e.l.i.s.a. protocol was used throughout.

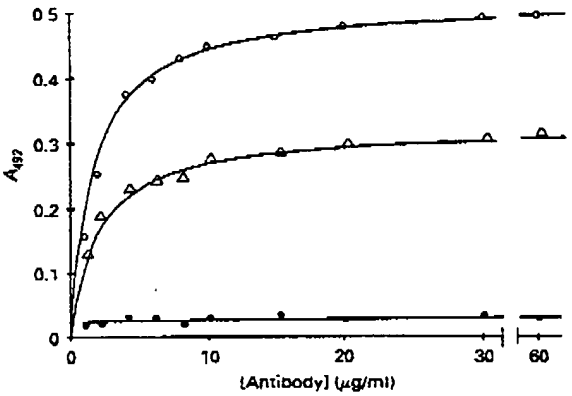

Fig 1. Indirect e.l.i.s.a. of intact (○), *N*-terminal (●) or *C*-terminal fragments (△) of CaM

Wells were coated with CaM and fragments of 2.5 μg/ml and 1.25 μg/ml respectively

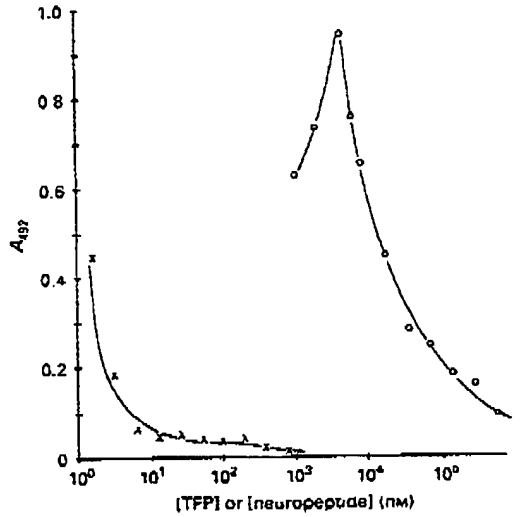

Fig 2. Effect of TFP (○) and the neuropeptide (×) on the binding of antibody to CaM

The coating concentration of CaM was 2.5 μg/ml and the antibody concentration was 2.5 μg/ml. The A_{492} value in the absence of antagonists was 0.511±0.039. For other details see the Materials and methods section.

Highly purified tryptic peptides of CaM have been obtained by h.p.l.c. Tryptic cleavage of CaM in the presence of Ca^{2+} results in two main fragments: CaM-(1–77) and CaM-(78–148) [14,16]. According to Guerini *et al.* [16], the two main fragments can be separated by reverse-phase h.p.l.c.: the *C*-terminal fragment with a retention time of 15.2 min as peptide CaM-(78–148) and the *N*-terminal fragment with a retention time of 17.4 min peptide CaM-(1–77) (Fig. 3). The retention time of intact CaM is very similar to that of the *N*-terminal fragment on this reverse-phase h.p.l.c. column (17.8 min). Fig. 3 shows the time-dependent elution profile of the tryptic digest of CaM. After 60 min of limited proteolysis, intact CaM could not be detected in the

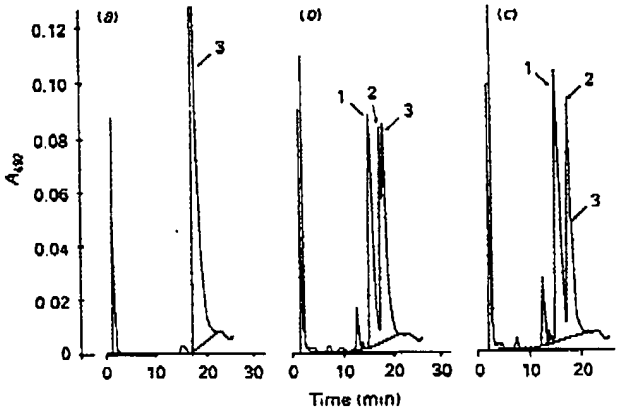

Fig 3. Purification of CaM fragments by reverse-phase h.p.l.c.

Chromatograms were performed with flow rate of 1 ml/min. A 20 μl sample of the crude proteolysate of CaM (2.0 mg/ml) was injected. The column was eluted with 10 mM-$(NH_4)_2CO_3$/acetonitrile (9:1, v/v) for 5 min, followed by an increasing linear acetonitrile gradient to a final concentration of 40% at 20 min. (*a*), (*b*) and (*c*) are the h.p.l.c. profiles of the CaM digestion mixture after 0, 30 and 60 min respectively. The peaks were identified as CaM (3), and its *C*-terminal (1) and *N*-terminal (2) fragments.

digested mixture (cf. Fig. 3). The homogeneity of the fragments was demonstrated by urea/PAGE gels.

The reactivity of *N*- and *C*-terminal fragments towards the monospecific antibody was investigated. As expected, the *N*-terminal fragment did not bind to the antibody (Fig. 1). This result also confirms that this fragment was not contaminated by intact CaM. The *C*-terminal fragment had a similar affinity for the antibody as intact CaM, but a lower plateau was observed with the fragment than with CaM, although the same molar concentrations were applied for immobilization. This may be due to a lower total or active concentration of this fragment bound to the solid phase.

Binding of TFP and neuropeptide to CaM fragments

We have investigated the effect of TFP on the binding of antibody to the solid-phase-bound *C*-terminal fragment and the mixture of tryptic fragments of CaM compared with intact CaM. Fig. 4 demonstrates that both the mixture of fragments and the *C*-terminal fragment have lost their ability to be stimulated by TFP in antibody binding.

A similar set of experiments was carried out with the neuropeptide (Fig. 5). While this peptide with potent anti-CaM activity displaced the antibody from intact CaM in the nanomolar concentration range, it failed to influence the binding of antibody to the *C*-terminal fragment of CaM in the same concentration range. As shown in Fig. 5, the peptide was effective only at concentrations two orders of magnitude higher than in the case of intact CaM.

The *N*-terminal fragment of CaM does not have antigenic site (cf. Fig. 1). To determine its neuropeptide-binding activity, two sets of competition e.l.i.s.a. experiments were carried out. In one set, the effect of neuropeptide on the formation of the immunocomplex was investigated in the presence of 3.2 μM *N*-terminal fragment. As shown in Fig. 6, at this concentration, which is about two orders of magnitude greater than that of intact CaM, the *N*-terminal fragment significantly neutralized the inhibitory effect of the neuropeptide on the binding of antibody to CaM.

In another set of experiments the concentration of *N*-terminal fragment was varied at a constant (50 nM) neuropeptide con-

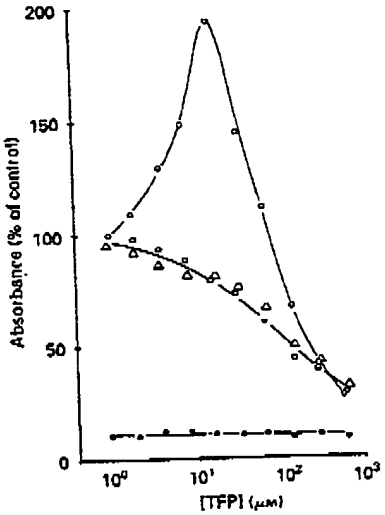

Fig 4. Effect of TFP on the formation of immunocomplex

The coating concentration of CaM (○) and its crude proteolysate (□) was 2.5 μg/ml, and that of the *C*-terminal (△) and *N*-terminal (●) fragments of CaM was 1.25 μg/ml. The antibody concentration was 2.5 μg/ml. For other details, see the Materials and methods section.

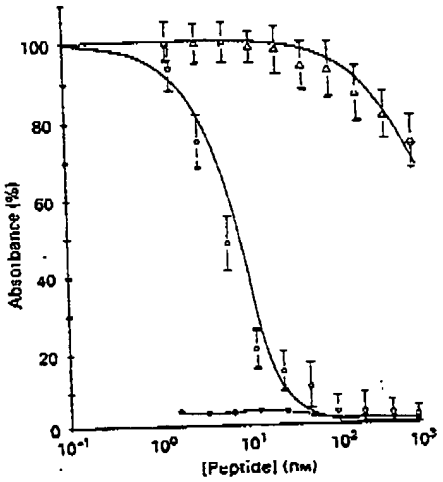

Fig 5. Displacement curve of the neuropeptide

The coating concentrations of intact CaM (○) and its *C*-terminal (△) or *N*-terminal (●) fragments was 2.5 μg/ml, the antibody concentration was 2.5 μg/ml. For other details see the Materials and methods section.

centration (Table 1). This pepude concentration abolished the formation of CaM–antibody complex in the absence of the *N*-terminal fragment (cf. Fig. 2). Table 1 shows that, with increasing fragment concentration, the inhibition of immunocomplex formation by neuropeptide decreased. This effect can be attributed to the binding of the neuropeptide to the fragment, which significantly decreases the concentration of CaM–neuropepude complex. Consequently the antibody binds to the intact CaM,

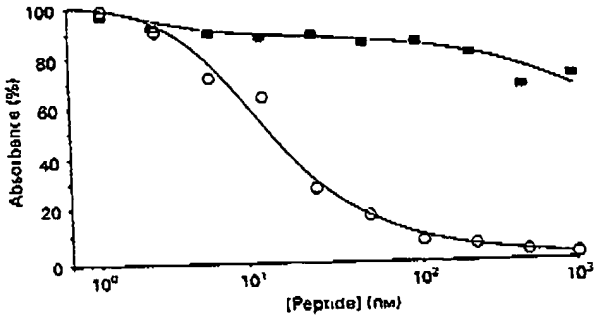

Fig 6. Effect of *N*-terminal fragment of CaM on the displacement curve of the neuropeptide

The coating concentration of intact CaM was 2.5 μg/ml; antibody concentration was 3.2 μg/ml. The concentrations of the fragment were 0 (○) or 3.2 μM (■), respectively. For other details see the Materials and methods section.

Table 1. Effect of *N*-terminal fragment of CaM on neuropeptide–CaM–antibody interactions

The amounts of CaM and antibody in the microtitre plate wells were 1.5 pmol and 2.1 pmol respectively. The wells were filled with 100 μl of solution. The amount of CaM was estimated according to [21]

[Neuropeptide] (pmol)	[*N*-Terminal fragment] (pmol)	Absorbance (%)
5	0	5±1
5	1.25	19±2
5	20	44±4
5	160	80±9
0	0	100±11

resulting in an increasing intensity in the colour reaction. A half-maximal effect of *N*-terminal fragment occurred at a concentration which is approx. one order of magnitude higher than that of intact CaM.

DISCUSSION

We have demonstrated previously the high sensitivity, simplicity and efficiency of the e.l.i.s.a. procedure for studying the effect of CaM antagonists on CaM–antibody interactions [10, 21]. In these indirect e.l.i.s.a. reactions an enzyme-conjugated anti-antibody is utilized in a colorimetric reaction as a measure of immunocomplex formation. The surface-bound CaM displayed similar affinity to antibody as did CaM in solution if the immobilization of CaM was carried out in the presence of Ca^{2+} [21]. Therefore the conformational changes in CaM structure caused by the immobilization process are probably not significant. In this paper we report that the native tertiary structure of CaM, probably the long α-helix structure exposed by Ca^{2+} binding, is absolutely necessary for the long-distance interactions between the *N*- and *C*-terminal domains.

The tertiary structure of CaM is well-established [28], and it is known that both the *N*- and *C*-terminal domains of CaM have one TFP-binding site [8]. CaM has a single antigenic site in the *C*-terminal region (residues 137–143) with which the monospecific antibody interacts [26,27]. Our experiments showed that TFP at low concentrations stimulates, whereas at relatively high concentrations it inhibits, immunocomplex formation. We interpret this complex binding character of TFP in the following way. The

binding of TFP to one of the binding domains stimulates the binding of antibody to CaM. However, the binding of TFP to the other domain is antagonistic to antibody binding, and at about 500 μM drug concentration the binding is decreased. Similar phenomena have been described by Newton *et al.* [29], who found that CaM conjugated with 1 mol of another phenothiazine, norchloropromazine, bound with high affinity to phosphodiesterase, erythrocyte Ca^{2+}–Mg^{2+}-ATPase, myosin light chain kinase, calcineurin, phosphorylase kinase and CaM-dependent kinase, and activated some of them. However, CaM conjugated with 2 mol of phenothiazine/mol of protein was completely inactive with regard to the binding or activation of any of these target proteins.

Another possibility should also be considered to explain the fact that the maximum colour reaction in the e.l.i.s.a. occurred at a given TFP concentration: one can hypothesize that the binding of TFP to either CaM or antibody alters the supramolecular organization of the CaM–antibody–(anti-antibody protein) complex. However, conclusions from further e.l.i.s.a. experiments using CaM fragments support the first explanation (cf. Fig. 4). Although the *C*-terminal fragment contains the monospecific antigenic site and thus displays similar affinity for the antibody as does CaM, when the mixture of the two halves of CaM was subjected to e.l.i.s.a. the stimulatory effect of TFP disappeared. These observations suggest that the native conformation of CaM has a specific role in the co-operative interaction between the TFP-binding site, probably localized in the *N*-terminal domain, and the antigenic site of CaM in the *C*-terminal domain.

A neuropeptide isolated from hypothalamus by Galoyan and his co-workers [9] inhibits the formation of the immunocomplex (cf. Fig. 2) and displaces the antibody from intact CaM at nanomolar concentrations [12]. The possibility that the peptide binds directly to the antibody as an analogue of a CaM fragment has been excluded [12]. Therefore it has been concluded that CaM is a potent target of neuropeptide binding.

To further characterize the neuropeptide-binding activity of CaM, e.l.i.s.a.s were performed using both *C*-terminal and *N*-terminal fragments of CaM. The direct test showed that the affinity of neuropeptide for the *C*-terminal fragment was lower by more than an order of magnitude in comparison with that for intact CaM. When the *N*-terminal fragment, which does not contain an antigenic site, was subjected to competition e.l.i.s.a. (competition of immobilized CaM and *N*-terminal fragment for neuropeptide binding), the inhibitory effect of the neuropeptide on immunocomplex formation was significantly decreased. The liberator effect of the *N*-terminal fragment depended on both the neuropeptide and the *N*-terminal fragment concentrations. The inhibitory effect of the neuropeptide was abolished if the concentration of the *N*-terminal fragment was about two orders of magnitude greater than that of CaM. Although the *N*-terminal fragment showed somewhat higher affinity for neuropeptide than did the *C*-terminal fragment, it is unclear how the intact CaM binds the neuropeptide with such high affinity. One possibility is that the two halves of the intact CaM share the binding of neuropeptide.

An extracellular peptide, melittin, shows several similarities to the neuropeptide in its binding to CaM and CaM fragments. For example, both peptides bind with similar affinity [21,30,31] to intact CaM. Melittin also forms complexes with both *N*- and *C*-terminal tryptic fragments, these interactions were three orders of magnitude smaller than that with intact CaM [17]. In addition, the sizes of melittin and the neuropeptide are similar (approx. 3 kDa). Melittin has been suggested to cross-link the two halves of CaM [7,32,33], bringing them sufficiently close for simultaneous binding. One can hypothesize that the hypothalamic neuropeptide interacts with intact CaM in a similar manner to melittin, the native conformation of CaM is essential for interaction with both peptides with very high affinity.

In conclusion, the complexity of the interaction of TFP with CaM, and the strong interaction of a neuropeptide with intact CaM, but not with its fragments, demonstrates that long-distance interactions exist within the native CaM structure. These long-distance interactions create effective communications between different binding domains of the intact CaM, e.g. between the TFP-binding domain in the *N*-terminal half and the antigenic site in the *C*-terminal half of the intact CaM. The effects produced by TFP and the neuropeptide on the formation of the immunocomplex are very different, which is not surprising since they have very different chemical structures. In the case of TFP the effect also depends on the concentration of this anti-CaM drug, which suggests the existence of different conformation states of CaM at different TFP saturation levels. These distinct conformers of CaM probably exhibit different activatory/inhibitory effects on the target enzymes.

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(54) Title: REVERSE TWO-HYBRID METHOD

(57) Abstract

The reverse two-hybrid method has been designed to provide a practical and efficient means of utilizing yeast cell-based assays to screen for molecules that can inhibit protein-protein interactions of interest. Existing two-hybrid systems involve reconstitution in yeast of a transcriptional activator that drives expression of a "reporter" gene such as *HIS3* or *lacZ*. Attempts to utilize these existing systems for drug discovery would necessarily involve screening for molecules that interfere with the transcriptional read-out, and would be subject to detecting any compound that non-specifically interfered with transcription. In addition, since currently used reporter genes encode long-lived proteins, the assay would have to be performed over a lengthy time period to allow for decay of the preexisting reporter proteins. Any compound that would be toxic to yeast over this time period would also score as a "hit". The reverse two-hybrid interaction will avoid both of these pitfalls by driving the expression of a relay gene, such as the *GAL80* gene, which encodes a protein that binds to and masks the activation domain of a transcriptional activator, such as Gal4. The reporter genes, which will provide the transcriptional read-out (*HIS3* or *lasZ*), are dependent upon functional Gal4 for expression. Only when the level of Gal80 masking protein is reduced by interfering with the two-hybrid interaction will Gal4 function as a transcriptional activator, providing a positive transcriptional read-out for molecules that inhibit the two-hybrid protein-protein interaction. An important feature of the reverse two-hybrid system is that the basal level and half-life of the relay protein, Gal80, can be fine-tuned to provide maximum sensitivity.

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REVERSE TWO-HYBRID METHOD

TECHNICAL FIELD

The invention relates to methods and compositions for identifying agents which modify intermolecular association between two or more polypeptides. Agents which specifically inhibit such protein-protein interactions are suitable for use as commercial reagents, pharmaceuticals, and for modulating gene expression in a cell culture and/or animal, such as to increase or decrease the expression of a predetermined protein in the cell culture or animal, and the like.

BACKGROUND

Specific protein-protein interactions are fundamental to most cellular and organismal functions. Polypeptide interactions are involved in formation of functional transcription complexes, signal transduction pathways, cytoskeletal organization (e.g., microtubule polymerization), polypeptide hormone receptor-ligand binding, organization of multi-subunit enzyme complexes, and the like.

Investigation of protein-protein interactions under physiological conditions has been problematic. Considerable effort has been made to identify proteins that bind to proteins of interest. Typically, these interactions have been detected by using co-precipitation experiments in which an antibody to a known protein is mixed with a cell extract and used to precipitate the known protein and any proteins which are stably associated with it. This method has several disadvantages, such as: (1) it only detects proteins which are associated in cell extract conditions rather than under physiological, intracellular conditions, (2) it only detects proteins which bind to the known protein with sufficient strength and stability for efficient co-immunoprecipitation, and (3) it fails to detect associated proteins which are displaced from the known protein upon antibody binding. For these reasons and others, improved methods for identifying proteins which interact with a known protein have been developed.

Two-Hybrid Systems

One approach has been to use a so-called "two-hybrid" system to identify polypeptide sequences which bind to a predetermined polypeptide sequence present in a fusion protein (Chien et al. (1991) Proc. Natl. Acad. Sci. (USA) 88: 9578). This approach identifies

protein-protein interactions in vivo through reconstitution of a transcriptional activator (Fields S and Song O (1989) Nature 340: 245), the yeast Gal4 transcription protein. The method is based on the properties of the yeast Gal4 protein, which consists of separable domains responsible for DNA-binding and transcriptional activation. Polynucleotides encoding two hybrid proteins, one consisting of the yeast Gal4 DNA-binding domain fused to a polypeptide sequence of a known protein and the other consisting of the Gal4 activation domain fused to a polypeptide sequence of a second protein, are constructed and introduced into a yeast host cell. Intermolecular binding between the two fusion proteins reconstitutes the Gal4 DNA-binding domain with the Gal4 activation domain, which leads to the transcriptional activation of a reporter gene (e.g., *lacZ*, *HIS3*) which is operably linked to a Gal4 binding site. Typically, the two-hybrid method is used to identify novel polypeptide sequences which interact with a known protein (Silver SC and Hunt SW (1993) Mol. Biol. Rep. 17: 155; Durfee et al. (1993) Genes Devel. 7: 555; Yang et al. (1992) Science 257: 680; Luban et al. (1993) Cell 73: 1067; Hardy et al. (1992) Genes Devel. 6: 801; Bartel et al. (1993) Biotechniques 14: 920; and Vojtek et al. (1993) Cell 74: 205). However, variations of the two-hybrid method have been used to identify mutations of a known protein that affect its binding to a second known protein (Li B and Fields S (1993) FASEB J. 7: 957; Lalo et al. (1993) Proc. Natl. Acad. Sci. (USA) 90: 5524; Jackson et al. (1993) Mol. Cell. Biol. 13; 2899; and Madura et al. (1993) J. Biol. Chem. 268: 12046). Two-hybrid systems have also been used to identify interacting structural domains of two known proteins (Bardwell et al. (1993) med. Microbiol. 8: 1177; Chakraborty et al. (1992) J. Biol. Chem. 267: 17498; Staudinger et al. (1993) J. Biol. Chem. 268: 4608; and Milne GT and Weaver DT (1993) Genes Devel. 7; 1755) or domains responsible for oligomerization of a single protein (Iwabuchi et al. (1993) Oncogene 8; 1693; Bogerd et al. (1993) J. Virol. 67: 5030). Variations of two-hybrid systems have been used to study the in vivo activity of a proteolytic enzyme (Dasmahapatra et al. (1992) Proc. Natl. Acad. Sci. (USA) 89: 4159). Alternatively, an E. coli/BCCP interactive screening system (Germino et al. (1993) Proc. Natl. Acad. Sci. (U.S.A.) 90: 933; Guarente L (1993) Proc. Natl. Acad. Sci. (U.S.A.) 90: 1639) can be used to identify interacting protein sequences (i.e., protein sequences which heterodimerize or form higher order heteromultimers).

Each of these two-hybrid methods rely upon a positive association between two Gal4 fusion proteins thereby reconstituting a functional Gal4 transcriptional activator which then induces transcription of a reporter gene operably linked to a Gal4 binding site. Transcription of the reporter gene produces a positive readout, typically manifested either (1)

as an enzyme activity (e.g., β-galactosidase) that can be identified by a colorimetric enzyme assay or (2) as enhanced cell growth on a defined medium (e.g., *HIS3*). Thus, these methods are suited for identifying a positive interaction of polypeptide sequences, but are poorly suited for identifying agents or conditions which alter (e.g., inhibit) intermolecular association between two polypeptide sequences. In part, this is because a failure to obtain expression of the reporter gene can result from many events which do not stem from a specific inhibition of binding of the two hybrid proteins. For example, a two-hybrid system using a reporter gene that stimulates growth under defined conditions theoretically can be used to screen for agents that inhibit the intermolecular association of the two hybrid proteins, but it will be difficult or impossible to discriminate agents that specifically inhibit the association of the two hybrid proteins from agents which simply inhibit cell growth. Thus, an agent which is cytotoxic to yeast (e.g., bleach, phenol, ketoconazole, cycloheximide) will prevent cell growth without specifically inhibiting the interaction of two hybrid proteins and will score falsely as a positive hit. Similarly, a conventional two-hybrid system using a *lacZ* reporter gene will falsely score general transcription or translation inhibitors (e.g., cycloheximide) as being inhibitors of two hybrid protein binding. Thus, two-hybrid systems that produce a positive readout contingent upon intermolecular binding of the two hybrid proteins are generally not suitable for screening for agents which inhibit binding of the two hybrid proteins.

Unfortunately, it would be desirable to have an efficient screening method for identifying compounds which specifically alter the intermolecular association between two known polypeptide sequences under physiological conditions. Present two-hybrid methods rely on a positive readout and do not afford a method for identifying binding inhibitors (or binding competitors) with satisfactory sensitivity and/or selectivity.

Thus, there is a need in the art for compositions and methods which can be used to efficiently identify agents that specifically alter the intermolecular association between two polypeptide sequences in vivo. The present invention fulfills these and other needs.

The references discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior invention. All publications and parent applications herein are incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

SUMMARY OF THE INVENTION

The present invention provides several novel methods and compositions for identifying agents which alter intermolecular binding between two polypeptide species in a cell or in a cell-free transcription reaction. The invention relates to a general method, referred to herein as a reverse two-hybrid method, wherein agents which disrupt an intermolecular association between two interacting polypeptides thereby generate a selectable and/or detectable readout (e.g., complementation of an auxotrophic phenotype, expression of a detectable reporter molecule, and the like). Typically, a reverse two-hybrid method produces a positive readout under conditions wherein an agent blocks or otherwise inhibits the intermolecular binding of the interacting polypeptides. A positive readout condition is generally identified as one or more of the following detectable conditions: (1) an increased transcription rate of a predetermined reporter gene, (2) an increased concentration or abundance of a polypeptide product encoded by a predetermined reporter gene, typically such as an enzyme which can be readily assayed in vivo, and/or (3) a selectable or otherwise identifiable phenotypic change in an organism (e.g., yeast) harboring the reverse two-hybrid system. Generally, a selectable or otherwise identifiable phenotypic change that characterizes a positive readout condition confers upon the organism either: a selective growth advantage on a defined medium, a mating phenotype, a characteristic morphology or developmental stage, drug resistance, or a detectable enzymatic activity (e.g., β-galactosidase, luciferase, alkaline phosphatase, and the like). In this manner, it is possible to efficiently identify agents (including but not limited to polypeptides, small molecules, and oligonucleotides) which inhibit intermolecular binding between two predetermined interacting polypeptides.

In an aspect of the invention, a reverse two-hybrid system is composed of: (1) a first hybrid protein, (2) a second hybrid protein which binds to the first hybrid protein under control conditions (e.g., physiological conditions in the absence of agent), (3) a relay (or signal inverter) gene which is efficiently expressed as a consequence of the first hybrid protein and the second hybrid protein being functionally bound to each other, and (4) a reporter gene which is efficiently expressed when the product of the relay (or signal inverter) gene is substantially absent and is either poorly expressed or not expressed when the relay (or signal inverter) gene product is efficiently expressed. The first hybrid protein and second hybrid protein bind to each other through interacting polypeptide segments (i.e., a portion of the first hybrid protein preferentially binds to a portion of the second hybrid protein forming a heterodimer or higher order heteromultimer comprising the first and second hybrid proteins; said binding portions of each hybrid protein are termed "interacting polypeptide segments").

The first hybrid protein comprises: (1) a first interacting polypeptide sequence in polypeptide linkage with (2) a DNA-binding domain of a transcriptional activator protein or other DNA binding protein (e.g., a repressor). The second hybrid protein comprises: (1) a second interacting polypeptide sequence, capable of forming an intermolecular association with the first interacting polypeptide sequence under control conditions (e.g., physiological conditions and absence of agent) in polypeptide linkage with (2) an activation domain of a transcriptional activator protein, whereby intermolecular binding between the first hybrid protein and the second hybrid protein (via the interacting polypeptide sequences) thereby unites the DNA-binding domain of the first hybrid protein with the activation domain of the second generating a transcriptional activator function. Generally, the first hybrid protein and the second hybrid protein are encoded by polynucleotides which are constitutively expressed in a host organism (e.g., a eukaryotic or prokaryotic cell, or multicellular organism).

The relay gene (alternatively termed the signal inverter gene) is operably linked to a transcriptional regulatory sequence (a "relay transcriptional regulatory sequence") which is positively regulated by the transcriptional activator that is formed by the intermolecular binding of the first hybrid protein to the second hybrid protein. Hence, when the first hybrid protein binds to the second hybrid protein (via the interacting polypeptide sequences), the transcriptional activator formed thereby binds to a transcriptional regulatory sequence operably linked to the relay gene and enhances the net transcription of the relay gene. The relay gene encodes a protein that represses transcription of a reporter gene. Thus, when the first and second hybrid proteins are functionally bound to each other, the relay gene is expressed and thereby represses transcription of the reporter gene(s). In an embodiment, such relay proteins are of the type often referred to in the art as "negative regulators of transcription". In an embodiment of the invention, the relay gene is a negative regulator of transcription in yeast; for example but not limitation the *GAL80* gene can serve as a relay gene in yeast. In embodiments where host organisms are employed to harbor the reverse two-hybrid system, the relay gene is often a gene which naturally occurs in the germline DNA of the host organism species, and frequently can be an endogenous germline gene, or alternatively may be introduced into the host organism as exogenous DNA, typically into a host genome that lacks the corresponding functional endogenous gene (e.g., a "knockout background").

The reporter gene is operably linked to a transcriptional regulatory sequence ("reporter transcriptional regulatory sequence") which is negatively regulated by the gene product of the relay gene and which is induced in the absence of the relay gene product.

Thus, transcription of the reporter gene is repressed in control conditions (e.g., physiological conditions in the absence of agent) wherein the two hybrid proteins bind to each other and form a transcriptional activator that increases transcription of the relay gene. Generally, the relay gene product either binds to the transcriptional regulatory sequence operably linked to the reporter gene, or binds to a transcription protein that binds to the transcriptional regulatory sequence operably linked to the reporter gene. The net transcription rate of the reporter gene is reduced (or completely blocked) as a consequence of the relay gene product binding to the reporter gene transcriptional regulatory sequence and/or to a transcription protein required for constitutive expression of the reporter gene. Any of a variety of reporter genes that produce a positive readout can be used. For example and not limitation, suitable reporter genes are those which (1) confer a selectable phenotype to cells in which the reporter gene is efficiently expressed, and/or (2) encode a gene product (e.g., enzyme) which is conveniently detected such as by in situ assay or the like. Suitable genes which confer a selectable phenotype are exemplified by, but not limited to, genes which complement auxotrophic mutations in a host organism (e.g., yeast *HIS3*), genes which encode drug resistance (e.g., neo^R), genes which induce cell proliferation, and other genes whose expression confers a selective growth advantage. Suitable genes which encode a gene product which is conveniently detected in situ are exemplified by, but not limited to, β-galactosidase (e.g., E. coli *lacZ*), luciferase, alkaline phosphatase, horseradish peroxidase, and the like.

The invention provides polynucleotides encoding a first hybrid protein and a second hybrid protein. Such polynucleotides encode a DNA-binding domain or activation domain of a transcriptional activator and conveniently can have a cloning site for adjacent insertion, in reading frame, of polynucleotide sequences encoding one or more interacting polypeptide sequence(s). Typically, a first polynucleotide will encode a first hybrid protein composed of a first predetermined interacting polypeptide sequence and a DNA-binding domain of a transcriptional activator; a second polynucleotide will encode a second hybrid protein composed of a second predetermined interacting polypeptide sequence and an activation domain of a transcriptional activator, wherein the DNA-binding domain of the first hybrid protein can reconstitute with the activation domain and form a functional transcriptional activator. Often, the DNA-binding domain and the activation domain of the hybrid protein pair are derived from the same naturally occurring transcription activator (e.g., Gal4). However, those of skill in the art can select DNA-binding domains and activation domains from distinct transcription activators which can reconstitute to form a functional transcriptional activator which does not occur in nature (e.g., a DNA-binding domain of the bacterial *lexA*

protein can be used in conjunction with a transcriptional activator from the viral protein, VP16; Vojtek et al. (1993) op.cit.). Transcription and translation of such a polynucleotide produces a hybrid (or fusion) protein composed of an interacting polynucleotide segment and a DNA-binding domain or activation domain of a transcriptional activator.

The invention also provides polynucleotides which comprise a transcriptional regulatory sequence operably linked to a relay (or signal inverter) gene. A relay (or signal inverter) gene encodes a protein that inhibits or otherwise represses expression (typically transcription) of a predetermined reporter gene. Most usually, a relay protein is a negative regulator of transcription for a predetermined gene or gene subset. In an embodiment, the relay protein is a transcription repressor protein that binds to a polynucleotide sequence and thereby inhibits transcription of a cis-linked and operably linked sequence. In an alternative embodiment, the relay protein binds to a protein that is a positive regulator of transcription of a predetermined gene or gene subset, and as a consequence of binding thereby inhibits the transcriptional activity of the positive regulator. One variety of such a relay protein binds to and blocks the activation domain(s) of transcriptional activators. Although a variety of suitable relay proteins are apparent to those of skill in the art, this category of relay protein can be exemplified by the mammalian *mdm2* oncoprotein which binds the transactivation domain of the tumor suppressor protein *p53*, and the yeast Gal80 protein which binds and inactivates the activation domain of Gal4. In an embodiment, the relay protein comprises a mutation, addition, or deletion that reduces the stability of the relay protein in vivo as compared to the naturally occurring cognate relay protein. Relay proteins can be referred to as signal inverter proteins, as they serve to invert a positive transcriptional signal (the reconstitution of a functional transcriptional activator by binding of the two hybrid proteins) into a negative transcriptional signal, which reduces transcription of a predetermined reporter gene. Generally, a polynucleotide encoding a relay protein is operably linked to a relay transcriptional regulatory sequence that produces transcription of the relay gene dependent upon functional reconstitution of the DNA-binding domain and activation domain of the two hybrid proteins. For example and not limitation, such a relay transcriptional regulatory sequence can comprise a promoter and a polynucleotide sequence comprising one or more site(s) which bind(s) a reconstituted functional transcriptional activator formed by association of the two hybrid proteins; for example, if the two hybrid transcriptional activator comprises a *lexA* DNA-binding domain, the relay transcriptional regulatory sequence operably linked to the relay gene can comprise one or more *lexA* binding site sequences, arrayed in tandem.

The invention also provides polynucleotides which comprise a transcriptional

regulatory sequence operably linked to a reporter gene. The reporter gene encodes a protein that confers a selectable phenotype on a host cell and/or can be detected by an in vivo assay, such as an in situ enzymatic assay (e.g., host cells expressing a *lacZ* reporter can be detected as blue staining cells in the presence of X-gal). A transcriptional regulatory sequence operably linked to the reporter gene comprises a promoter and generally produces constitutive transcription of the relay gene contingent upon the substantial absence of the relay protein. In an embodiment, the reporter transcriptional regulatory sequence operably linked to the reporter gene comprises a binding site for a relay protein, wherein binding of the relay protein to the reporter transcriptional regulatory sequence inhibits constitutive transcription of the reporter gene. In an alternative embodiment, the reporter transcriptional regulatory sequence linked to the reporter gene comprises a binding site for a transcriptional activator protein, wherein binding of a constitutive transcriptional activator protein to the reporter transcriptional regulatory sequence produces constitutive transcription of the cis-linked reporter gene, and wherein the relay protein binds to or otherwise inactivates the transcriptional activator protein, thereby repressing constitutive expression of the reporter gene.

The invention also provides host organisms (typically unicellular organisms) which harbor a reverse two-hybrid system, typically in the form of polynucleotides encoding a first hybrid protein, a second hybrid protein, a relay gene, and/or a reporter gene, wherein said polynucleotide(s) are either stably replicated or introduced for transient expression. In an embodiment, the host organism is a yeast cell (e.g., Saccharomyces cervisiae) in which the germline *GAL80* gene is functionally inactivated, the relay gene encodes Gal80, and the reporter gene transcriptional regulatory sequence comprises a Gal4-responsive promoter.

The invention also provides a method for identifying agents that inhibit binding of a first interacting polypeptide to a second interacting polypeptide. The method employs the reverse two-hybrid system described *supra*, wherein a first hybrid protein comprises the first interacting polypeptide and a second hybrid protein comprises a second interacting polypeptide. Heterodimerization (or higher order heteromultimerization) between the first hybrid protein and the second hybrid protein produces transcription of a relay gene encoding a protein which inhibits expression of a reporter protein. Host organisms harboring such a reverse two-hybrid system are cultured in the presence of an agent, such as a diffusible small molecule (typical MW $< 5{,}000$, preferably $< 1{,}000$) or a transfected cDNA expression polynucleotide encoding a polypeptide agent, and expression of the host organism reporter gene is determined and standardized to a parallel blank culture which lacks an agent. Agents which produce a gnificant increase in expression of the reporter gene in a host organism

after a suitable time period (e.g., usually at least 1 hour, often at least 3 hours, preferably about 6 hours, occaisionally overnight or longer) are thereby identified as inhibitors for blocking the intermolecular association between the first an second interacting polypeptide sequences. Such protein interaction inhibitors are candidate drugs for pharmaceutical use and/or for use as commercial research reagents. In an embodiment of the invention, yeast cells are the host organism, the reporter gene encodes β-galactosidase and/or a protein that complements an auxotrophic mutant yeast host cell, and the first and second interacting polypeptide sequences each comprise a binding domain derived from a signal transduction protein.

The invention also provides a kit comprising a reverse two-hybrid system, a host cell, and an instruction manual. Such kits may optionally include a panel of agents for testing.

DETAILED DESCRIPTION

Definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described. For purposes of the present invention, the following terms are defined below.

In the polypeptide notation used herein, the lefthand direction is the amino terminal direction and the righthand direction is the carboxy-terminal direction, in accordance with standard usage and convention. Similarly, unless specified otherwise, the lefthand end of single-stranded polynucleotide sequences is the 5' end; the lefthand direction of double-stranded polynucleotide sequences is referred to as the 5' direction. The direction of 5' to 3' addition of nascent RNA transcripts is referred to as the transcription direction; sequence regions on the DNA strand having the same sequence as the RNA and which are 5' to the 5' end of the RNA transcript are referred to as "upstream sequences"; sequence regions on the DNA strand having the same sequence as the RNA and which are 3' to the 3' end of the RNA transcript are referred to as "downstream sequences".

The term "naturally-occurring" as used herein as applied to an object refers to the fact that an object can be found in nature. For example, a polypeptide or polynucleotide sequence that is present in an organism (including viruses) that can be isolated from a source

in nature and which has not been intentionally modified by man in the laboratory is naturally-occurring.

The term "agent" is used herein to denote a chemical compound, a mixture of chemical compounds, a biological macromolecule, or an extract made from biological materials such as bacteria, plants, fungi, or animal (particularly mammalian) cells or tissues. Agents are evaluated for potential activity as specific protein interaction inhibitors (i.e., an agent which selectively inhibits a binding interaction between two predetermined polypeptides but which does not substantially interfere with cell viability) by inclusion in screening assays described hereinbelow.

The term "protein interaction inhibitor" is used herein to refer to an agent which is identified by one or more screening method(s) of the invention as an agent which selectively inhibits protein-protein binding between a first interacting polypeptide and a second interacting polypeptide. Some protein interaction inhibitors may have therapeutic potential as drugs for human use and/or may serve as commercial reagents for laboratory research or bioprocess control. Protein interaction inhibitors which are candidate drugs are then tested further for activity in assays which are routinely used to predict suitability for use as human and veterinary drugs, including in vivo administration to non-human animals and often including administration to human in approved clinical trials.

As used herein, the term "operably linked" refers to a linkage of polynucleotide elements in a functional relationship. A nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For instance, a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the coding sequence. Operably linked means that the DNA sequences being linked are typically contiguous and, where necessary to join two protein coding regions, contiguous and in reading frame. However, since enhancers generally function when separated from the promoter by several kilobases and intronic sequences may be of variable lengths, some polynucleotide elements may be operably linked but not contiguous.

As used herein, the term "endogenous DNA sequence" refers to naturally-occurring polynucleotide sequences contained in a eukaryotic or prokaryotic cell. Such sequences include, for example, chromosomal sequences (e.g., structural genes, promoters, enhancers, recombinatorial hotspots, repeat sequences, integrated proviral sequences). A "predetermined sequence" is a sequence which is selected at the discretion of the practitioner on the basis of known or predicted sequence information. An exogenous polynucleotide is a

polynucleotide which is transferred into a eukaryotic or prokaryotic cell.

As used herein the term "physiological conditions" refers to temperature, pH, ionic strength, viscosity, and like biochemical parameters which are compatible with a viable organism, and/or which typically exist intracellularly in a viable cultured yeast cell or mammalian cell. For example, the intracellular conditions in a yeast cell grown under typical laboratory culture conditions are physiological conditions. Suitable in vitro reaction conditions for in vitro transcription cocktails are generally physiological conditions. In general, in vitro physiological conditions comprise 50-200 mM NaCl or KCl, pH 6.5-8.5, 20-45°C and 0.01-10 mM divalent cation (e.g., Mg^{++}, Ca^{++}); preferably about 150 mM NaCl or KCl, pH 7.2-7.6, 5 mM divalent cation, and often include 0.01-1.0 percent nonspecific protein (e.g., BSA). Particular aqueous conditions may be selected by the practitioner according to conventional methods. For general guidance, the following buffered aqueous conditions may be applicable: 10-250 mM NaCl, 5-50 mM Tris HCl, pH 5-8, with optional addition of divalent cation(s) and/or metal chelators and/or nonionic detergents and/or membrane fractions.

The terms "functional disruption" or "functionally disrupted" as used herein means that a gene locus comprises at least one mutation or structural alteration such that the functionally disrupted gene is substantially incapable of directing the efficient expression of functional gene product.

As used herein, the terms "interacting polypeptide segment", and "interacting polypeptide sequence" refer to a portion of a hybrid protein which can form a specific binding interaction with a portion of a second hybrid protein under suitable binding conditions. Generally, a portion of the first hybrid protein preferentially binds to a portion of the second hybrid protein forming a heterodimer or higher order heteromultimer comprising the first and second hybrid proteins; the binding portions of each hybrid protein are termed interacting polypeptide segments.

Description of the Invention

Generally, the nomenclature used hereafter and the laboratory procedures in cell culture, molecular genetics, and nucleic acid chemistry and cell culture described below are those well known and commonly employed in the art. Standard techniques are used for recombinant nucleic acid methods, polynucleotide synthesis, and microbial culture and transformation (e.g., electroporation, lipofection). Generally enzymatic reactions and purification steps are performed according to the manufacturer's specifications. The

techniques and procedures are generally performed according to conventional methods in the art and various general references (see, generally, Sambrook et al. Molecular Cloning: A Laboratory Manual, 2d ed. (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., which is incorporated herein by reference) which are provided throughout this document. The procedures therein are believed to be well known in the art and are provided for the convenience of the reader. All the information contained therein is incorporated herein by reference.

The reverse two-hybrid method is generally applicable for identifying agents which inhibit binding between a variety of predetermined interacting polypeptides.

Overview

A basis of the present invention is a strategy for screening a bank of agents with a reverse two-hybrid system to identify agents which inhibit the intermolecular association of two interacting polypeptide sequences. Thus, in a reverse two-hybrid system there is at least one pair of interacting polypeptide sequences, with a first interacting polypeptide sequence present in one of the hybrid protein species and a second interacting polypeptide sequence present in the other hybrid protein species. The choice of interacting polypeptide sequences incorporated in a reverse two-hybrid system is selected at the discretion of the practitioner. For example, a reverse two-hybrid system suitable for identifying agents which inhibit *Fos/Jun* leucine zipper formation may be composed of a first hybrid protein having an interacting polypeptide sequence comprising a *Fos* leucine zipper and a second hybrid protein having an interacting polypeptide sequence comprising a *Jun* leucine zipper. A variety of interacting protein sequences can be used; for example and not limitation, these include: transcription factor binding domains, multisubunit proteins, signal transduction proteins (G proteins, members of *ras/raf/MEK* signaling pathway(s), tumor suppressor protein binding domains (*Rb*, *p53*), and the like), polypeptide ligands and their cognate receptor(s), active sites of enzymes which catalyze reactions involving binding to a polypeptide substrate and the polypeptide substrate itself, and essentially any pair of protein sequences which form an intermolecular association under physiological conditions. Generally, interacting polypeptides form heterodimers with a dissociation constant (K_d) of at least about 1×10^{3} M^{-1}, usually at least 1×10^{4} M^{-1}, typically at least 1×10^{5} M^{-1}, preferably at least 1×10^{6} M^{-1} to 1×10^{7} M^{-1} or more, under suitable physiological conditions.

Reverse two-hybrid systems are used for detecting the ability of agents to inhibit the intermolecular binding of two interacting polypeptides and provide for facile high-

throughput screening of agent banks (e.g., compound libraries, peptide expression libraries, and the like) to identify protein interaction inhibitors which preferentially inhibit intermolecular binding between two predetermined interacting polypeptide species. Such protein interaction inhibitors (specific binding antagonists) can modulate biochemical activity of the predetermined interacting specie(s) and thereby modulate biological function. Agents which alter the intermolecular association of the two interacting polypeptide sequences in the hybrid proteins, generally by inhibiting heterodimeric binding of the two hybrid proteins, score positively in the reverse two-hybrid system. The protein interaction inhibitors thereby identified are candidate drugs for human and veterinary therapeutic use and/or are suitable commercial reagents for laboratory research or bioprocess control.

An agent capable of specifically inhibiting protein-protein binding of a therapeutically relevant protein interaction in vivo can be used for therapy of disease or for modulation of gene expression in cells and organisms. Typically, an efficacious dose of a protein interaction inhibitor is administered to a patient as a therapeutic or prophylactic for treating a pathological condition (e.g., cancer, inflammation, lymphoproliferative diseases, autoimmune disease, and the like).

Description of the Preferred Embodiment

In order to illustrate the invention, a description of a preferred embodiment is presented below. This embodiment comprises a reverse two-hybrid system in yeast cells that are functionally disrupted for endogenous *GAL80* expression, wherein the intermolecular association of the first and second hybrid proteins activates transcription of a *GAL80* relay gene. Expression of *GAL80* represses the transcriptional activity of constitutively expressed Gal4 protein and inhibits transcription of a Gal4-dependent reporter gene.

A variety of alternative embodiments and variations will be apparent to those of skill in the art, including alternative relay genes, alternative host cells (e.g., mammalian, bacterial, fungal, insect, and the like), variations of the basic reverse two-hybrid method, and others.

Two-Hybrid systems

Transcriptional activators are proteins that positively regulate the expression of specific genes. They can be functionally dissected into two structural domains: one region that binds to specific DNA sequences and thereby confers specificity, and another region termed the activation domain that binds to protein components of the basal gene expression

machinery (Ma and Ptashne (1988) Cell 55: 443). These two domains need to be physically connected in order to function as a transcriptional activator. Two-hybrid systems exploit this finding by hooking up an isolated DNA binding domain to one protein (protein X), while hooking up the isolated activation domain to another protein (protein Y). When X and Y interact to a significant extent, the DNA binding and activation domains will now be connected and the transcriptional activator function reconstituted (Fields and Song (1989) Nature 340: 245). The yeast host strain is engineered so that the reconstituted transcriptional activator drives the expression of a specific reporter gene such as *HIS3* or *lacZ*, which provides the read-out for the protein-protein interaction (Field and Song (1989) op.cit.; Chein et al. (1991) op.cit.). One advantage of two-hybrid systems for monitoring protein-protein interactions is their sensitivity in detection of physically weak, but physiologically important, protein-protein interactions. As such it offers a significant advantage over other methods for detecting protein-protein interactions (e.g., ELISA assay). Unlike the ELISA assay, however, the current two-hybrid system is not readily transplantable to drug screening operations. A major problem with the existing two-hybrid methods is that nonspecific inhibitors of transcriptional activation score the same as inhibitors of the specific protein-protein interaction.

Negative Regulators of Transcription

To address the aforementioned problem, the read-out of the conventional two-hybrid interaction can be reversed by interposition of a relay gene which serves to invert the output produced from interaction of the two hybrid proteins from a positive transcriptional activator to a negative transcriptional regulator (e.g., repressor). In order to invert the readout from a positive transcription activator to a negative transcription repressor, it is possible to take advantage of the properties of certain negative regulators of transcription. In an embodiment, some of these negative regulators block the function of specific transcriptional activators by binding to their activation domain. Two such examples are the *mdm2* oncoprotein which binds to and masks the trans-activation domain of the tumor suppressor protein *p53* (Momand et al. (1993) Cell 69: 1237; Oliner et al. (1993) Nature 362: 857), and the yeast Gal80 protein which binds and inactivates the transcriptional activator region of Gal4 (Ma and Ptashne (1987) Cell 50: 137; Johnston and Carlson (1993) Regulation of Carbon and Phosphate Metabolism, vol. 2, Cold Spring Harbor Press, Cold Spring Harbor, New York). By designing the two-hybrid interaction to drive the expression of a negative regulator of a specific transcriptional activator, the resultant system is such that interference with the two-hybrid interaction results in increased activity of a transcriptional activator and hence a

positive signal. In view of the fact that the biology of the Gal80-Gal4 system is well understood in yeast, this pair of negative-regulators/transcriptional activators is suitable for the reverse two-hybrid method. In principle, the pair of *mdm2* and *p53* proteins, or any other matched pair of transcriptional activator and specific negative regulator, will also work.

In the present embodiment, the two interacting hybrid proteins reconstitute a transcriptional activator composed of a DNA binding domain derived from the bacterial protein encoded by *lexA* and an activator domain derived from the viral protein *VP16* (Vojtek et al. (1993) op.cit.). The reconstituted *lexA/VP16* transcriptional activator binds to a relay gene operably linked to a transcriptional regulatory sequence containing tandem copies of a *lexO* binding site sequence which binds the *lexA* DNA-binding domain. Upon binding of the reconstituted *lexA/VP16* transcriptional activator to the *lexO* binding site(s), the operably linked relay gene (*GAL80*) is efficiently expressed. Thus, when the two hybrid proteins are associated (e.g., as a heterodimer or the like), the *GAL80* relay gene is expressed and serves to repress expression of a reporter gene construct.

The Gal80-Gal4 System

The Gal80-Gal4 system of regulatory proteins underlies the ability of yeast cells to respond to exogenously added galactose and specifically synthesize the enzymes needed to utilize it as a carbon/energy source (Johnston and Carlson (1993) op.cit.. Unless galactose is present, the Gal80 protein binds and blocks the function of the transcriptional activator Gal4. In the absence of the *GAL80* gene, the transcriptional activator function of Gal4 is not masked and hence expression of galactose-regulated genes no longer requires galactose for induction. In the reverse two-hybrid system, the host strain generally is functionally disrupted for the endogenous *GAL80* gene, but Gal80 protein is provided through a two-hybrid driven relay gene construct (see, Experimental Example, *infra*) which is operably linked to a transcriptional regulatory sequence that binds a bacterial lexA DNA-binding domain present in the first hybrid protein. When the two-hybrid interaction is driving the expression of the relay gene product, Gal80, the Gal4-induction of the reporter gene(s) is inhibited. When the two-hybrid interaction is blocked, the relay gene (*GAL80*) expression will be turned off and the Gal4-dependent transcriptional regulatory sequence operably linked to the reporter gene(s) is then able to drive expression of the reporter gene(s).

Techniques for Fine-Tuning Expression of the Relay Gene

The sensitivity of this system can be modulated by adjusting the amount of

Gal4 or Gal80 protein. A host strain generally contains the wild-type *GAL4* gene and hence contains very low levels of Gal4 when the yeast cells are cultured with carbon/energy sources such as raffinose (Johnston and Carlson (1993) op.cit.). If necessary, the level of Gal4 protein can be decreased by at least five-fold by culturing the cells in glucose (Griggs and Johnston (1991) Proc. Natl. Acad. Sci. (USA) 88: 8597). Higher levels of Gal4 protein can be provided by transforming the strain with a multicopy plasmid encoding Gal4 (Schultz et al. (1987).

The amount and/or stability of the relay protein, Gal80, can also be adjusted. Preferably, the stability of the Gal80 protein is sufficient such that the addition of protein interaction inhibitor agents generates a detectable readout of the reporter gene(s) within about six hours, or most usually within the time-frame of an overnight assay. For this to be a convenient assay approach, Gal80 activity preferably deteriorates at a rapid rate when active inhibitor agents are added and the two-hybrid system is inhibited. The half-life of Gal80 proteins in yeast cells has not been rigorously defined in the art. If Gal80 has a short half-life, it is generally only necessary to vary the level of transcription of *GAL80* by changing either copy number of the two-hybrid relay gene construct or by varying the number of binding sites for the transcriptional activator (e.g., *lexO* operator sequences) in the transcriptional regulatory sequence of the relay gene construct. If Gal80 has an inordinately long half-life, it is preferable to engineer a chimeric Gal80 protein with a shorter half-life. Successful engineering of long-lived proteins to proteins with shorter half-lives has been achieved by addition of PEST sequences to *DHFR* (Loetscher et al. (1991) J. Biol. Chem. 266:11213) or by forming β-galactosidase variants with different N-terminal residues by in vivo processing of ubiquitin-β-galactosidase fusions (Varshavsky et al. (1989) Yeast Genetic Engineering, Barr, Brake, and Valenzuela (eds.), Butterworths, pp. 109-143). The latter method has been well characterized in yeast, such that Gal80 variants with half-lives ranging from 2 minutes to over 24 hours can be readily generated.

The following examples are offered by way of example and not by way of limitation.

EXPERIMENTAL EXAMPLES

Construction of the appropriate host yeast strains

Since the GAL80-GAL4 system is employed, the reporter genes in the yeast strain need to be operably linked to promoters that are responsive to Gal4. Reporter genes that have been operably linked to Gal4-responsive promoters were integrated into yeast strains (see Construction of Yeast Strains, *infra*). One of the reporter genes encodes β-galactosidase,

whose expression allows quantitative transcriptional read-out, if desired. It is possible to utilize other reporter genes operably linked to Gal4-responsive promoters, such as ones encoding alkaline phosphatase, that would also allow easy quantitation of transcriptional read-out. JEY8, JEY10, and JEY12, three independent progenitors for the reverse-two hybrid host strains, were derived by standard genetic methods from a cross between YM2170 (*MATa ura3 his3 ade2 lys2 tyr1 GAL4⁺ gal80Δ LEU2::GAL1-lacZ*; available from Dr. Mark Johnston, Washington University, St. Louis, MO) and YPB2 (*MATa his3 ade2 leu2 ura3 lys2 trp1 can1 gal4Δ gal80Δ LYS2::GAL1-HIS3 URA3::GAL1-lacZ*) (Bartel et al. (1993) in Cellular Interactions in Development: A Practical Approach, Hartley DA (ed.) Oxford University Press, Oxford, UK, pp. 153). The progenitor strains (*MATa his3 ade2 leu2 ura3 lys2 trp1 GAL4⁺ gal80Δ LYS2::GAL1-HIS3 URA3::GAL1-lacZ*) contain all the necessary reporter genes and have been tested for a functional Gal4 protein and reporter genes by analysis of galactose-induced expression of β-galactosidase. To test in general whether re-introduction of Gal80 protein negatively regulates Gal4 in the system, JEY8 was transformed with a high-copy plasmid containing the wild-type *GAL80* gene (pBM260; available from Mark Johnston, Washington University, St. Louis, MO). Sufficient expression results in inhibition of the read-outs from the reporter genes (*HIS3* and *lacZ*), which are determined by assaying β-galactosidase activity and growth in the absence of histidine. Both of these reporter activities are scored in yeast grown on plates containing raffinose (which allows for full activity of Gal80 protein) and galactose (which inactivates Gal80 protein). These tests confirm that in these strains the Gal80 protein, expressed off its endogenous promoter, suppresses Gal4 function. Two-hybrid constructs are evaluated for their ability to drive sufficient *GAL80* expression from the *LexO-GAL80* fusion plasmids that are constructed (see, *infra*).

Construction of the LexO-GAL80 fusion genes

A chimeric gene (a *LexO-GAL80* fusion) is constructed and serves as the relay (signal inverter) gene. The DNA-binding domain of the transcriptional activator that is used to drive expression of the relay gene is derived from the bacterial protein encoded by *lexA* and has been used before in two-hybrid systems as a fusion with the transcriptional activator from the viral protein VP16 (Vojtek et al. (1993) op.cit.. Other transcriptional activators that have a defined DNA binding site, such as the *ACE1* gene product of *S. cerevisiae* (Munder and Furst (1992) Mol. Cell. Biol. 12: 2091) may be used. The *LexO* sites are generated by mutually primed synthesis (see, Chapter 8.2A in Current Protocols in Molecular Biology (1990) Ausubel, Brent, Kingston, Moore, Seidman, Smith, and Struhl (eds.), Greene

Publishing Associates and Wiley Interscience, New York, NY) using the oligomer 5'-GCGAATTCCTACTGTATATACATACAGTACCATCTACTGTATATACATACAGTAGC CGCTCGAGCGGC-3' [SEQ. ID NO:1]. The resulting fragment contains four consensus *LexA* binding sites in tandem. The DNA product is digested with EcoRI and inserted into the EcoRI site of pCZD (Lue et al. (1989) Proc. Natl. Acad. Sci. (USA) 86: 486) to generate pCZD-LexO. The pCZD vector contains a minimal TATA box for recognition of the basal transcriptional machinery but requires the addition of specific DNA sequences to effectively function as promoter box. The Gal80 coding sequence is isolated by PCR using the following two oligomers: 5'-CGCGGATCCCGTTCTTTCCAC TCCCG-3' [SEQ. ID NO:2]; and 5'-CGGATCCGATGGAAGGATGCCCGCTGCTGC-3' [SEQ. ID NO:3]. The template is the plasmid pBM260 which contains the *GAL80* gene subcloned in YEp13 (available from Mark Johnston, Washington University, St. Louis, MO). The *GAL80* PCR product is digested with BamHI and inserted into the BamHI site of pCZD-LexO to create pLexO-Gal80. The *LexO-Gal80* fusion is then subcloned into pGalileo, a 2μ based yeast shuttle vector (20-30 copies per cell) carrying the *ADE2* selectable marker (available from Avtar Roopra, Washington University, St. Louis, MO) to generate pJE42. From this plasmid, CEN- and integrating versions are constructed to provide a means of controlling the level of expression of *GAL80* by the two-hybrid interaction. For example, the basal transcription from the 2μ plasmid may express sufficient Gal80 to require galactose for expression of the reporter gene(s) even in the absence of a lexA-based transcriptional activator. Additionally, LexO-ubiquitin-Gal80 fusions encoding a shortened half-life Gal80 protein is constructed.

In order to demonstrate that relay gene constructs comprising a *LexO-GAL80* polynucleotide fusion can be activated by the two-hybrid interaction to sufficient levels for regulating the Gal4-mediated reporter gene expression, a positive control is generated. Yeast are transformed with a plasmid that contains a fusion of the DNA binding domain (lexA) and transcriptional activation component (VP16) of the two-hybrid system and activates transcription of the relay gene *LexO-GAL80* fusion. The plasmid pLEX-VP16 (available from A. Vojtek; Vojtek et al. (1993) op.cit.) is used for the positive control. The ability of two-hybrid interactions to drive expression of the relay gene is demonstrated.

Testing for the ability of two-hybrid interactions to drive expression of the LexO-GAL80 fusions

The two-hybrid interaction that is used to test for its ability to drive sufficient

expression of the *LexO-GAL80* relay gene is the interaction of human H-ras p21 with human c-Raf (Van Aelst et al. (1993) Proc. Natl. Acad. Sci. (USA) 90: 6213; Vojtek et al. (1993) op.cit.). K-ras is linked by in-frame polynucleotide fusion to the VP16 activation domain, and Raf is linked by in-frame polynucleotide fusion to the DNA binding domain of the *lexA* gene product. pGBT8-Raf was constructed by ligating EcoRI and PstI linkers to a *Raf* coding sequence isolated by PCR amplification of a human placental cDNA library from Stratagene (San Diego, CA) as described by MacDonald et al. (1993) Mol. Cell. Biol. 13: 6615. The *Raf* gene was cut out of pGBT8-Raf as an EcoRI to PstI fragment and subcloned into the EcoRI-PstI site of pBTM116 (that contains the LexA DNA binding domain (Vojtek et al. (1993) op.cit.) to generate pBTM-Raf (pJE36). The EcoRI site maintains the same reading frame. pGBT8K-ras was constructed by PCR amplification of pEXV-K-ras (Hancock et al. (1990) Cell 63: 133) such that the K-ras sequence is isolated as a SalI-PstI restriction fragment which was then subcloned into SalI-PstI-cut pGBT8. To construct pVPK-ras (pJE44), a PCR product of pGBT8K-ras was generated using the following oligomers as PCR amplimers:

5'-CGGGATCCATGACTGAATATAAACTTGTGGTAG-3' [SEQ. ID NO:4]

5'-CGGGATCCTTACATAATTACACACTTTGTCTTTCACTTG-3' [SEQ. ID NO:5]

and the resultant PCR product was digested with BamHI and subcloned into the BamHI site of pVP16 (Vojtek et al. (1993) op.cit.) to generate pVPK-ras (pJE44). The *LexO-GAL80* relay gene plasmid, the pBTM-Raf and the pVP-K-ras (pJE44) plasmids are cotransfected into a host yeast strain and the ability of the two-hybrid interaction to drive sufficient expression of *GAL80* to prevent the expression of the reporter genes (*lacZ* and *HIS3*) is determined. Growth on galactose is used as an internal positive control to ensure that the promoter is still functional.

Testing for the ability of a small molecule to interfere with a two-hybrid interaction

The reverse two-hybrid method is used as a screening assay for identifying small molecule inhibitors of protein-protein interaction, such that an exogenously added small molecule can interfere with a two-hybrid interaction. In one example, a reverse two-hybrid system utilizes the small molecule estradiol as the protein interaction inhibitor. Estradiol is a small lipophilic molecule that has been shown to be effective in yeast. It has been shown that estradiol reverses the interaction of the hormone binding domain of the estrogen receptor with the heat-shock protein HSP90. Thus, a first hybrid protein comprising the hormone binding domain of the estrogen receptor in polypeptide linkage to a lexA DNA-binding domain and a second hybrid protein comprising the heat shock protein, HSP90, in polypeptide linkage to the

VP16 activation domain are constructed by standard methods. Polynucleotide(s) encoding the first and second hybrid proteins, a *LexO-GAL80* relay gene construct, and a Gal4-dependent reporter gene construct are introduced into the yeast host. Estrogen (e.g., estradiol) is evaluated as an agent for inhibiting formation of a functional two-hybrid heterodimer and thereby producing expression of the reporter gene.

Testing for the ability of a polypeptide to interfere with a two-hybrid interaction

The reverse two-hybrid method is used as a screening assay for identifying polypeptide inhibitors of protein-protein interaction, such that an intracellularly expressed polypeptide can interfere with a two-hybrid interaction. In one embodiment, a reverse two-hybrid system utilizes a polypeptide expressed from a cotransfected cDNA expression construct as the protein interaction inhibitor.

A first hybrid protein comprising a first interacting polypeptide sequence in polypeptide linkage to a lexA DNA-binding domain and a second hybrid protein comprising a second interacting polypeptide sequence in polypeptide linkage to the VP16 activation domain are constructed by standard methods. Polynucleotide(s) encoding the first and second hybrid proteins, a *LexO-GAL80* relay gene construct, and a Gal4-dependent reporter gene construct are introduced into the yeast host. A polynucleotide encoding and expressing a polypeptide typically between 5 and 500 amino acids long (e.g., a library member of a cDNA expression library) is also introduced into the yeast cells under conditions wherein the encoded polypeptide is expressed intracellularly. The expressed polypeptide is evaluated as an agent for inhibiting formation of a functional two-hybrid heterodimer and thereby producing expression of the reporter gene.

Essentially any of various expression clone libraries known in the art may be used, including commercially available expression libraries (Clontech, Inc., Palo Alto, CA). Expression clone libraries may also be generated by the practitioner by conventional cloning methods and vectors known in the art (e.g., pcD, pSV), especially yeast expression vectors. Expression clone libraries comprise a collection of library members, each member comprising a cloned polynucleotide sequence (which may comprise mutation(s) or deletions), typically a cDNA sequence, operably linked to a promoter (and optionally an enhancer) which is transcriptionally active in the host cell so that the cloned sequence is transcribed and translated into a polypeptide. Genomic DNA sequences (e.g., complete structural genes or fragments thereof) may also serve as cloned sequences in expression libraries. Preferably, the cloned sequence is inserted in cloning site which facilitates the recovery of the cloned sequence free

from the promoter and other sequences which comprise an expression cassette.

Expression clone library members are transferred into host cells by various means, including but not limited to: electroporation, lipofection, viral vector transduction, biolistics, and $CaPO_4$ precipitation. Expression clone library members may be transferred directly into host cells, or a relay and/or reporter polynucleotide and/or polynucleotide(s) encoding the first and second hybrid proteins may be co-transferred with expression clone library members into a host cell, or a relay and/or reporter polynucleotide and/or polynucleotide(s) encoding the first and second hybrid proteins may be transferred into host cells subsequent to transfer of expression clone library members.

Cloned polynucleotides can be recovered from expression clone library members which are isolated by the screening methods of the invention. Typically, cloned sequences are excised by restriction digestion with an enzyme(s) which cleave at the boundaries between the ends of the cloned sequence (e.g., cDNA) and the remainder of the expression clone library member. Alternatively, PCR (preferably high-fidelity PCR) or other amplification method (e.g., LCR) may be performed using primers which flank the site at which the cloned sequence is inserted in the library member to amplify and thereby isolate the cloned sequence (U.S. Patent 4,683,202, incorporated herein by reference). When PCR is used, it is generally preferable to incorporate known unique polynucleotide sequences flanking at least one, and preferably both, side(s) of the site in which a cloned sequence is inserted to facilitate recovery of the selected cloned sequence(s).

Although the present invention has been described in some detail by way of illustration for purposes of clarity of understanding, it will be apparent that certain changes and modifications may be practiced within the scope of the claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: ERICKSON, JAMES R.
POWERS, SCOTT

(ii) TITLE OF INVENTION: REVERSE TWO-HYBRID METHOD

(iii) NUMBER OF SEQUENCES: 5

(iv) CORRESPONDENCE ADDRESS:
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(B) STREET: Five Palo Alto Square, Fourth Floor
(C) CITY: Palo Alto
(D) STATE: California
(E) COUNTRY: US
(F) ZIP: 94306-2155

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: Paremin Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/218,933
(B) FILING DATE: 29-MAR-1994
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Torchia, Timothy E.
(B) REGISTRATION NUMBER: 36,700

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (415) 843-5481

(B) TELEFAX: (415) 857-0663

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 68 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:

(A) NAME/KEY: misc_feature

(B) LOCATION: 1..68

(D) OTHER INFORMATION: /standard_name= "PCR primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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CGAGCGGC 68

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..26
(D) OTHER INFORMATION: /standard_name= "PCR primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CGCGGATCCC GTTCTTTCCA CTCCCG 26

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..30
(D) OTHER INFORMATION: /standard_name= "PCR primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CGGATCCGAT GGAAGGATGC CCGCTGCTGC 30

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..33
(D) OTHER INFORMATION: /standard_name= "oligomer for PCR"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CGGGATCCAT GACTGAATAT AAACTTGTGG TAG 33

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..39
(D) OTHER INFORMATION: /standard_name= "oligomer for PCR"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CGGGATCCTT ACATAATTAC ACACTTTGTC TTTCACTTG 39

CLAIMS:

1. A reverse two-hybrid system comprising:

(1) a first hybrid protein comprising a first interacting polypeptide sequence in polypeptide linkage to a DNA-binding domain of a transcriptional activator;

(2) a second hybrid protein comprising a second interacting polypeptide sequence in polypeptide linkage to an activation domain of a transcriptional activator, wherein the second hybrid protein binds to the first hybrid protein via contact of the interacting polynucleotide sequences under physiological conditions;

(3) a relay gene whose transcription is dependent upon the first hybrid protein and the second hybrid protein being bound to each other, thereby reconstituting a transcriptional activator; and

(4) a reporter gene whose transcription is repressed by expression of the relay gene and which is substantially transcribed in the absence of relay gene expression.

2. A reverse two-hybrid system of claim 1, wherein the two-hybrid system is in a yeast host cell.

3. A reverse two-hybrid system of claim 1, wherein the first hybrid protein, the second hybrid protein, a relay protein, and a reporter protein are encoded on at least one polynucleotide and said at least one polynucleotide is introduced into a yeast host cell.

4. A reverse two-hybrid system of claim 1, wherein the first hybrid protein comprises a lexA DNA-binding domain in polypeptide linkage to the first interacting polypeptide sequence;

the second hybrid protein comprises a VP16 activation domain in polypeptide linkage to the second interacting polypeptide sequence;

the relay gene encodes Gal80 operably linked to a LexO sequence in a cis-linked relay gene transcription regulatory sequence; and

the reporter gene comprises *lacZ* or *HIS3* and is operably linked to a transcription regulatory sequence which confers Gal4-dependent transcription to cis-linked adjacent polynucleotide sequences.

5. A reverse two-hybrid system of claim 4 in a yeast cell produced by crossing a Saccharomyces organism having the genotype *MATa his3 ade2 leu2 ura3 lys2 trp1 GAL4⁺ gal80D LYS2::GAL1-HIS3 URA3::GAL1-lacZ.*

6. A reverse two-hybrid system of claim 4 further comprising an agent.

7. A reverse two-hybrid system of claim 4 further comprises an expression clone library member which expresses an intracellular polypeptide in the yeast host.

8. A yeast cell comprising a reverse two-hybrid system of claim 1 or 4.

9. A polynucleotide encoding a Gal80 polypeptide and comprising at least one operably linked *LexO* binding site.

10. A polynucleotide of claim 9 in a yeast cell which contains a functionally disrupted endogenous *GAL80* gene.

11. A yeast cell containing:

a polynucleotide sequence encoding a first hybrid protein which is constitutively expressed;

a polynucleotide sequence encoding a second hybrid protein which is constitutively expressed;

a polynucleotide sequence encoding a relay protein whose expression is dependent upon intermolecular binding of the first hybrid protein with the second hybrid protein under physiological conditions; and

a polynucleotide sequence encoding a reporter protein whose expression is repressed by the relay protein.

12. A yeast cell of claim 11, further comprising an expression clone library member which expresses a polypeptide encoded by a cDNA.

13. A yeast cell of claim 11, further comprising an agent having a molecular weight of less than 1,000 daltons.

14. A method for identifying agents which inhibit intermolecular binding under physiological conditions between a first interacting polypeptide sequence and a second interacting polypeptide sequence, said method comprising the steps of:

administering an agent to a host cell containing a reverse two-hybrid system of claim 1 or 4 and incubating the host cell for a suitable period;

determining whether the administration of the agent induces a substantially expression of the reporter gene; and

identifying an agent which induces a substantially expression of the reporter gene as a protein interaction inhibitor.

15. A method of claim 14, wherein the agent is a molecule having a molecular weight less than about 1,000 daltons.

16. A kit comprising a reverse two-hybrid system, a host cell, an instruction manual, and optionally a panel of agents for testing.

17. A reverse two-hybrid system of claim 4, wherein the first interacting polypeptide sequence is a mammalian ras polypeptide and the second interacting polypeptide sequence is a Raf polypeptide.

INTERNATIONAL SEARCH REPORT

International application No
PCT/US95/03918

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 15/00, 15/06, 15/11, 15/12, 15/81
US CL : 435/7.1, 240.1, 254.21; 536/23.1, 23.2
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.1, 69.1, 69.9, 172.3, 240.2, 243, 320.1; 935/37, 43254.21; 536/23.1, 23.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Cell, Volume 74, issued 16 July 1993, A.B. Vojtek et al, "Mammalian Ras interacts directly with the serine/threonine kinase Raf", pages 205-214, see entire document.	1-17
A	Proceedings of the National Academy of Sciences, USA, Volume 88, issued December 1991, H.A. Vasavada et al, "A contingent replication assay for the detection of protein-protein interactions in animal cells", pages 10686-10690, see entire document.	1-17
A	Nature, Volume 340, issued 20 July 1989, Fields et al, "A novel genetic system to detect protein-protein interactions", pages 245-246, see entire document.	1-17

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 JUNE 1995	10 JUL 1995
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer JAMES MARTINELL Telephone No. (703) 308-0196

Form PCT/ISA/21 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/03918

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

ENTREZ 16.0: two-hybrid, two, hybrid, yeast, Saccharomyces, gal..., reverse..., gal..., lex...
APS: two, hybrid, 435/clas, 935/clas, 800/clas, gal80, gal(w)80, lexo, lex(w)o

Box 1. The two-hybrid system: an historical perspective

The yeast two-hybrid system relies on the unexpected finding that many protein domains retain some detectable function when grafted onto another unrelated protein domain. By the mid-1980s, cutting and religating DNA fragments had become routine – one fragment behaved much like another and all examples of a given restriction site were generally compatible – but it had not been considered productive to attempt similar strategies for proteins, with their complex and unique three-dimensional structures. While it was clear that some proteins could fold into independent domains that could carry out different functions, ligating together domains from two unrelated proteins was not common practice.

In studies of the regulation of transcription in eukaryotes, however, the idea that site-specific activators have a highly modular structure began to emerge. Findings from three types of experiments laid the foundations for the development of the two-hybrid system.

First, two separate functional domains of these transcriptional activators were characterized (see Figure, part a): for example, work in Ptashne's and in Struhl's laboratories identified the DNA-binding domains of the yeast Gal4 and Gcn4 proteins[35,36]. However, expression in yeast and binding of these isolated domains to their DNA recognition sites, called upstream activation sequences (UASs), was not sufficient to activate transcription of an adjacent gene. Rather, for transcription to occur, the DNA-binding domain had to be covalently linked to another portion of the protein that was highly acidic. This second domain was termed the transcription activation domain, and was defined as a sequence that when fused to a DNA-binding domain resulted in transcription. It was proposed that the activation domain interacts with components of the basal transcription machinery as part of the process of initiation of transcription.

Second, Ptashne's group showed that a hybrid activator could be generated[37], constructing a yeast plasmid that encoded a fusion of the *E. coli* LexA protein to sequences from the yeast Gal4 protein. The LexA–Gal4 fusion activated transcription in yeast of a reporter gene with LexA binding sites cloned upstream (see Figure, part b), while LexA alone did not. This experiment was later repeated by others with several different activators, with the general result that various combinations of a DNA-binding domain covalently linked to an activation domain could efficiently activate transcription.

Third, the concept of site-specific activators composed of two discrete proteins became accepted (see Figure, part c). Proteins were identified that interacted with DNA-binding proteins. One model proposed was that as a result of protein–protein interactions, these new proteins provided an activation function to the DNA-bound proteins, without themselves contacting DNA. Two such proteins were c-Fos and the *Herpes virus* protein VP16 (Refs 38, 39), it was later shown that these two proteins did in fact contact DNA, although they did not bind well by themselves. Nevertheless, this concept was critical to the origination of the two-hybrid system.

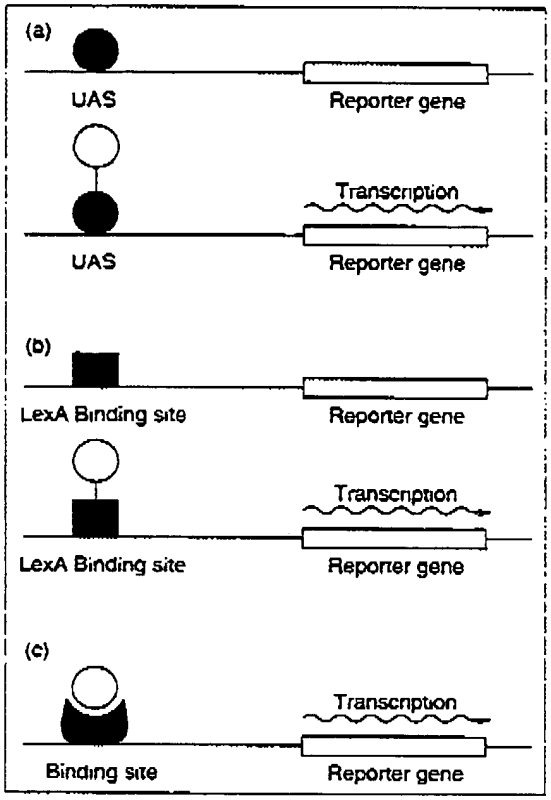

Figure. Key experiments that indicated the modular nature of transcription factors. (a) A DNA-binding domain (filled circle) can bind to an upstream activation sequence (UAS), but will not activate transcription. The DNA-binding domain covalently linked to a transcriptional activation domain (open circle) will activate transcription. (b) The LexA protein (filled square) when produced in yeast can bind to its operator site, but will not activate transcription. When LexA is covalently linked to an activation domain, the hybrid protein will activate transcription. (c) A transcriptional activator may be composed of two noncovalently linked proteins; one (filled circle) binds to a specific site on the DNA and the other (open circle) provides an activation domain.

While the two-hybrid system is most often used in yeast, it should work in any eukaryote, and has been used in a mammalian host (see, for example, Ref. 4). However, the yeast-based system has numerous advantages, including the ease of transformation, the convenience of retrieving plasmids, and the availability of nutritional markers and well-characterized reporter genes for direct selection. Finally, endogenous yeast proteins are less likely to bind a mammalian target protein to prevent its interaction with a protein encoded by a library.

Why the two-hybrid system works

Several features of the two-hybrid system make it applicable to a wide variety of proteins. First, many X–Y interactions analysed do in fact occur in yeast, thus the typical dissociation constants for these protein–protein interactions and the concentration of the hybrid proteins in yeast is such that binding can result. Second, many non-nuclear proteins can be targeted to the nucleus. Examples of proteins successfully used in two-hybrid assays include those normally found in the nucleus, cytoplasm or mitochondrion, and

membrane-associated and extracellular proteins. It is not known whether the hybrid proteins first interact within the nucleus or interact in the cytoplasm and are transported into the nucleus. Third, most X–Y interactions can result in transcriptional activity. The fact that so many pairs of hybrid proteins lead to reporter gene expression argues strongly that activation does not depend on there being a defined distance or orientation between the transcription factor domain and the fused X or Y domain. Thus the hybrid proteins might behave as two balls on a short flexible string rather than as two rigidly constrained domains.

The two-hybrid system is highly sensitive, and can detect interactions not revealed by other methods. For example, interaction of the mammalian Ras protein with the protein kinase Raf was observed in the two-hybrid system[5], but had not been detected by co-immunoprecipitation. It seems likely that transient interactions can trigger transcription to produce a stable mRNA that can be repeatedly translated to yield a reporter protein. This type of amplification leads to a detectable signal even when the initiating interaction cannot be observed *in vitro*. Other interactions with the basal transcription machinery may help stabilize a weak X–Y interaction. The sensitivity of this method suggests that certain enzyme–substrate interactions can be detected; for example, the combination of the protein kinase Raf and the IκB protein, which can be phosphorylated by Raf, yields a signal in this system[6].

Is transcriptional activity in the two-hybrid system proportional to affinity? This question can be addressed by assaying the activity of mutant versions of X or Y that differ at single residues and bind to the partner protein with differing affinities. For the p53–SV40 large T antigen combination, a good correlation was observed between transcription and affinity[7]. Affinity may be the major variable in determining the amount of transcriptional activity, the concentration of proteins, extent of nuclear import, accessibility of the X–Y domains to each other, and accessibility of the activation domain to the transcription machinery may play more minor roles. It may, therefore, be possible with a defined pair of proteins to establish dissociation values for novel mutants of one protein by first generating a standard curve based on biochemically determined K_D values of existing mutants. This should be feasible as long as the mutant proteins all have similar stabilities.

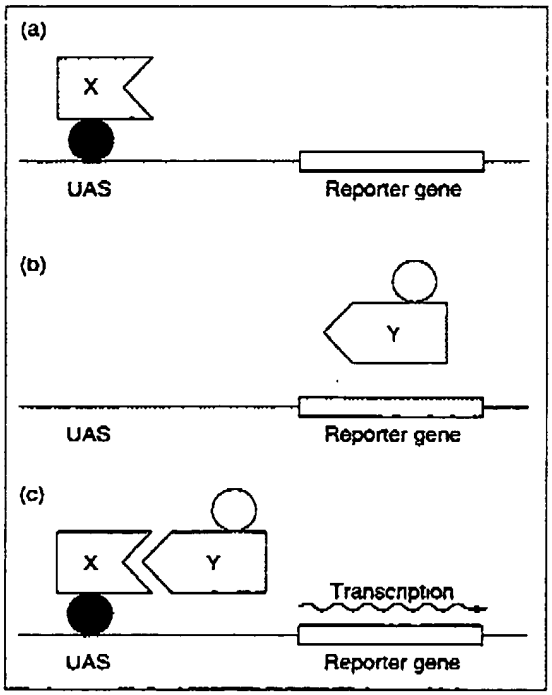

FIGURE 1. Outline of the two-hybrid system. (a) A hybrid protein is generated that includes a DNA-binding domain (filled circle) and a protein X. This hybrid can bind to DNA but will not activate transcription if X does not have an activation domain. (b) Another hybrid protein is generated that fuses an activation domain (open circle) to a protein Y. This hybrid protein will not activate transcription because it does not bind to the upstream activation sequence (UAS). (c) Both hybrid proteins are produced in the same transformant. The X and Y proteins bind noncovalently and activate transcription from the UAS.

Applications

The two-hybrid system has three major applications: testing known proteins for interaction, defining domains or amino acids critical for an interaction, and screening libraries for proteins that bind some target protein. Known proteins may be considered likely to interact with each other on the basis of genetic or biochemical data, physiological experiments, sequence similarities, or other criteria. If the genes that encode them are cloned, hybrids can be generated in which all or part of the proteins are fused to DNA-binding or activation domains. Hybrids may be designed to include domains based on structural features of a protein, to initiate from a convenient restriction site within a gene, or to include an entire coding sequence. The plasmids are introduced into a yeast reporter strain and assayed, generally by the production of β-galactosidase activity from a *lacZ* reporter gene. Examples of defined combinations of yeast proteins that function in this system include both the G_α–G_β and G_β–G_γ subunits, which function in pheromone response[8], the splicing factors Prp9–Spp91 (Ref. 9) and the mitochondrial proteins Pet54–Pet122 (Ref. 10). Examples from mammalian cells include oncoprotein or tumor suppressor combinations, such as Jun–Fos (Ref. 11), p53–p53 (Ref. 12) and Rb–SV40 large T antigen[13]; proteins involved in signal transduction, such as Sos–Grb2 (Ref. 14); proteins involved in transcription, such as TATA-binding protein (TBP)–TBP-associated factor $hTAF_{II}250$ (Ref. 15); and viral proteins, such as human immunodeficiency virus (HIV) Gag–Gag (Ref. 16).

If an interaction is detected, the domains of interaction can be delineated by making suitable deletions in the genes encoding X and Y and introducing plasmids with these deletions into the reporter strain. For example, a 192-residue domain in the p85 subunit of phosphatidylinositol-3-kinase was found to interact with the p110 subunit of this enzyme[17]. Holt *et al*[17] used analogous deletions of p110 to identify a 127-residue domain that can bind to p85. Once specific interacting domains are identified, the two-hybrid assay can

be used to identify residues critical for the interaction. For example, residues important for the interaction of p53 with SV40 large T antigen were identified by mutagenesis of small domains of the p53 protein followed by blue/white *lacZ* screening[7]. Many p53 mutants that failed to interact with T antigen were identified, and these correspond to sites that are changed in human cancers.

Perhaps the most powerful current application of the two-hybrid system is in identifying proteins that interact with a given target protein fused to a DNA-binding domain. Typically, libraries are constructed in which total cDNA derived from an organism or tissue is fused to a sequence encoding a transcription activation domain. These libraries generally contain $>10^6$ inserts, although only one-sixth of these are likely to be in the correct orientation and reading frame. Both oligo dT- and random-primed cDNA libraries have been generated, as well as size-selected libraries that encode only small protein domains.

In a two-hybrid search, it is critical that the hybrid protein containing the DNA-binding domain and the target protein does not itself produce any transcriptional activity, otherwise, it is likely that artefactual positives will be produced by, for example, increases in the copy number of the DNA-binding domain plasmid or stabilization of the DNA-binding domain hybrid (see below). It is best to perform library searches in a strain that contains both a selectable reporter, such as *HIS3*, and a second reporter that results in a color, such as *lacZ* (Ref. 13). If the plasmid encoding the DNA-binding domain hybrid confers no selective disadvantage, it can be transformed first, before retransformation with the activation domain library. Alternatively, the DNA-binding domain plasmid and the library are cotransformed, selecting for *HIS3* transcription by plating on media lacking histidine. Colonies are then analysed for β-galactosidase activity by transferring them to a filter that is placed in a solution of the chromogenic substrate X-Gal. Strains with dual reporter genes have several advantages. First, the selectable reporter allows a higher plating density than is feasible with a color screen. Second, the use of two reporter genes whose promoters are similar only at sites required for interaction with the DNA-binding domain can eliminate many false positives[18]. Third, continuous histidine selection throughout subsequent procedures eliminates irrelevant library plasmids that may have been cotransformed with the plasmid of interest. Moreover, plasmids that are toxic to the yeast can undergo rearrangements or mutations that eliminate expression of the activation domain hybrid, cells carrying these mutant plasmids tend to be lost as they do not survive under selection. Fourth, the β-galactosidase activity of transformants under *HIS3* selection is generally enhanced; this is probably because the transformants must produce the hybrid proteins in quantities that enable the *HIS3* gene to be expressed at levels that permit growth, and *lacZ* transcription is also increased as a consequence.

Library searches have now yielded proteins that interact with a variety of target proteins[2,12,13,19–24]. In studies of proteins involved in regulation of the cell cycle, the cyclin-dependent kinase Cdk2 was used to identify a 21 kDa protein[25], Cip1, and the related kinase Cdk4 detected a protein of 16 kDa[26]; these small proteins inhibit the catalytic activity of the kinases and may play a major role in tumorigenesis[27]. Cdk2 also identified a new protein, Rbr2, which is related to the retinoblastoma protein[28], and the cyclin-dependent kinase Cdc2 detected a protein phosphatase Cdi1 (Ref. 29). The transcriptional regulator Yin-Yang-1 was found to bind to c-Myc (Ref. 30), and Bcl2 to R-Ras (Ref. 31).

An extension of this technology is the one-hybrid system, used to identify proteins that bind a specific DNA sequence[32,33]. It is used when the DNA sequence bound by a protein is known, but the gene encoding the protein has not been isolated. Two components are required: a library, identical to those used in the two-hybrid approach, of random proteins fused to a transcription activation domain; and a reporter gene regulated by the specific sequence. The yeast strain used must not express the reporter gene, indicating that the DNA sequence of interest is not bound by any endogenous activator. Introduction of the library and selection and/or screening for reporter gene expression identifies plasmids encoding the desired DNA-binding domains. This method was first used to clone the gene for an olfactory neuronal transcription factor[32] and more recently for a component of the yeast origin recognition complex[33]. It appears to work well for non-abundant DNA-binding proteins, whose biochemical purification is difficult.

Problems and pitfalls

The two-hybrid system cannot assay all protein–protein interactions. Proteins that cannot fold correctly within the cell or be imported into the nucleus are clearly not suitable. Although interaction of an antibody with a protein antigen has been demonstrated in this system (P. Bartel and S. Fields, unpublished), it may not be possible to reproduce interactions involving other extracellular proteins: many are glycosylated and/or contain disulfide bonds, modifications not generally compatible with a nuclear-based system. Thus, the assay may be of limited use in analysing receptor–ligand interactions that usually occur outside the cell; however, it is likely that the intracellular domains of membrane receptors will function in the two-hybrid system.

Similarly, interactions that are mediated by post-translational modification, such as phosphorylation, may not be detected. For example, proteins with SH2 domains require phosphotyrosine in the proteins to which they bind, and this phosphorylation may not occur in yeast. However, it might be possible to phosphorylate a target protein so that it can interact with SH2-containing proteins by coexpressing a tyrosine-specific protein kinase. In general, although certain proteins may not be modified as they would be in animal cells, proteins produced in this eukaryotic system are probably more like their native state than those produced in bacterial systems.

If the protein–protein interaction is mediated by an amino-terminal domain of either protein, the standard orientation of the hybrid constructs may not work because the transcription factor domain blocks accessibility; in such cases, the orientation of the hybrid can be reversed. Proteins that activate transcription when fused to a DNA-binding domain can also cause problems

Box 2. Prospects for future applications of the two-hybrid system

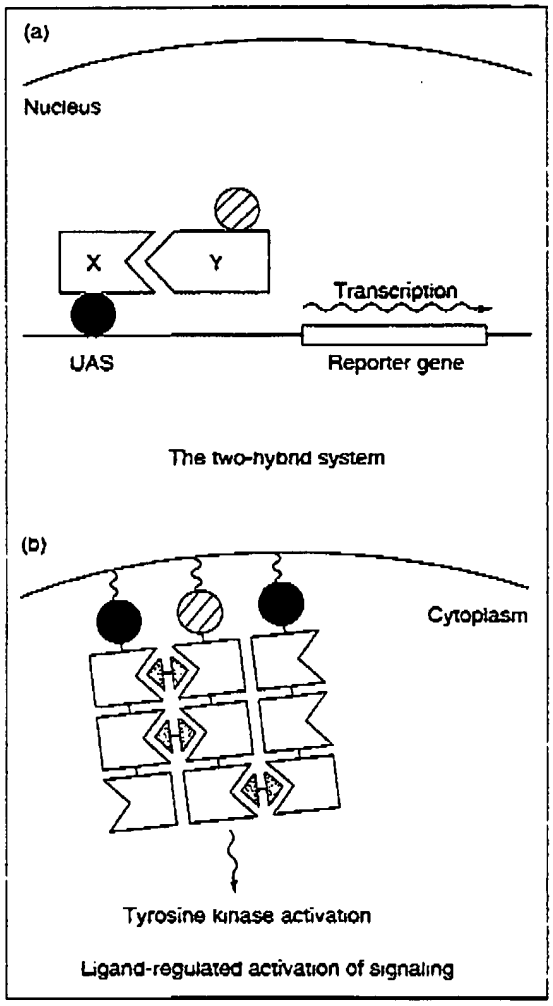

Figure. Comparison of the two-hybrid system and a method that allows inducible regulation of signal transduction. (a) The two-hybrid system, in which the interaction of two proteins X and Y causes transcription by bringing the transcription factor domains (filled and hatched circles) into proximity. (b) A system in which signal transduction is controlled using synthetic ligands. Hybrid proteins are induced to interact because they contain FKBP12 domains (open shapes), which bind the ligand FK1012 (double-headed arrows). This leads to the aggregation of T cell receptor ζ domains (shown as filled and hatched circles to make the analogy with the two-hybrid system) and, consequently, activation of the receptor and downstream signaling.

Additional applications of the two-hybrid system are currently being developed. Here, we describe its use in analysing protein–peptide interactions and in schemes for drug discovery. Extending the system to search for all possible interactions might lead to the generation of 'protein linkage maps'. Finally, we discuss an analogous system that allows inducible regulation of signal transduction.

The two-hybrid system can be used to assay interactions where one component is a small peptide. When the Rb protein is fused to the Gal4 DNA-binding domain, a 13-residue peptide that is derived from SV40 large T antigen and contains the Leu–X–Cys–X–Glu motif found in several Rb-binding proteins is sufficient to activate transcription when fused to the Gal4 activation domain (T. Durfee, B. Gorovits, C. Hensey and W-H. Lee, submitted; M. Yang and S. Fields, unpublished). On this basis, a library was constructed in which synthetically generated oligonucleotides encoding a 16-residue peptide are fused to the Gal4 activation domain (M. Yang and S. Fields, unpublished). This was searched for Rb-binding peptides and several peptides containing the Leu–X–Cys–X–Glu sequence were identified. Thus, such a library might be used to identify novel peptides, in much the same way as phage display systems are used. These could then form the basis for synthesizing peptides that have therapeutic potential.

A second potential use of the two-hybrid system in drug design is in identifying small molecules that inhibit a protein–protein interaction. This would involve making a yeast strain that produced an easily detected reporter protein, such as luciferase, as the result of a two-hybrid interaction. The X and Y proteins assayed would be targets for drug intervention, such as viral proteins. Screening this strain with various compounds would identify those that inhibit the X–Y interaction by binding to one of the proteins, as detected by decreased reporter gene expression. Such an approach has several advantages: it is an *in vivo* assay, and any inhibitor must cross the cell membrane to have an effect; however, unlike in other *in vivo* assays that rely on, for example, inhibition of viral growth, in this screen, the target of any inhibitor would be clearly defined.

Given the diversity of protein interactions that are detected in the two-hybrid system, this technique might be feasible on a genome-wide basis. The idea is to generate random libraries of both DNA-binding domain and activation domain hybrids, and to screen these against each other to detect a large number of interactions. This might allow protein A to be identified as binding to protein B, B as binding to C, and so on, ultimately allowing the construction of a 'protein linkage map'. This strategy may be applicable to entire simple organisms or to complex structures of other organisms. It might allow one-dimensional sequences generated from genome sequencing projects to be converted into a more three-dimensional view of protein–protein interactions.

The logic of using the interaction of two hybrid proteins can be extended to analyse other fundamental cellular processes. The principle of the two-hybrid system is that noncovalent interaction of two proteins triggers a process (transcription) in which these proteins normally play no role, because of their covalent linkage to domains that function in this process. In a novel method described by Schreiber and Crabtree[40], intracellular oligomerization of cell surface receptors was effected by linking them to an immunophilin, FKBP12 (see Figure). A ligand, FK1012, could bind two FKBP12 domains, causing aggregation of intracellular T cell receptor ζ domains and initiating downstream signaling events. In this method, the inducible noncovalent interaction of two proteins (both the X and Y components of the system are FKBP12) triggers a process (signal transduction) because of their covalent linkage to domains that function in this process (ζ domains). Other strategies using hybrid proteins may be designed to study additional biological phenomena.

In some cases, activation reflects the true function of a protein in transcription, but in many cases random proteins or protein domains can cause activation[24]. If such a protein is being tested in a defined combination with a known partner, it can be used instead as the activation domain partner. However, if it is used in a library search, the residues responsible for transcriptional activation must first be deleted, it is often impossible to tell what effect this might have on its ability to bind other proteins.

In assays of defined protein combinations, one orientation of the hybrids (i.e. X fused to the DNA-binding domain rather than to the activation domain) often activates transcription much more efficiently. This may reflect differences between the expression or stability of hybrids containing X and those containing Y. Transcription is optimal when the activation domain hybrid is in excess over the DNA-binding domain hybrid. When the reverse is true, DNA-binding domain hybrids bound to the UAS are less likely to be engaged in the X–Y protein–protein interaction. In some cases, as deletions of one hybrid are constructed and assayed to define a minimal domain for interaction, transcription increases significantly. While in certain instances this may be because residues that are not available in the intact protein are exposed in a shorter domain, it might also be that smaller proteins or domains simply work better in this system.

In library searches, false positives do occur: certain plasmids activate reporter gene expression but do not encode a protein that binds the target protein. These false positives are often caused by the library plasmid encoding a protein involved in transcription; in other cases, it is unclear how the encoded protein activates transcription. Certain false positives are particularly puzzling. With these plasmids, reporter transcription depends on the target protein moiety being present in the DNA-binding domain hybrid; when only the DNA-binding domain is present, these plasmids cannot activate transcription[18]. To rule out such false positives, plasmids that come through the initial selection and screening steps must be tested with hybrids of the DNA-binding domain and unrelated proteins. A rapid genetic test for this has been described[25]. In any event, false positives do not appear to be caused by non-specific binding of proteins to numerous other proteins.

Finally, interaction of the target and library-encoded proteins does not necessarily indicate that they normally interact *in vivo*. A protein family, such as the family of leucine zipper proteins, may have many members. While two of these may produce a signal in the assay, they may never normally be present in the same cell type, or cellular compartment, or during the same stage of the cell cycle. Similarly, the two-hybrid system may assay an interaction between domains that are not accessible in the native protein, particularly when an interaction is mediated via a short sequence, such as the interaction of the Rb protein with a Leu–X–Cys–X–Glu motif. If this motif occurs in a normally non-exposed domain of a protein that does not bind Rb, it may produce a signal when the isolated domain is specified by a hybrid vector. Of course, other methods for detecting protein–protein interactions can also yield artefactual signals, and interactions detected in these and in the two-hybrid system should be confirmed by biological or biochemical experiments.

Concluding remarks

Over the past five years, the usefulness of the two-hybrid system for detecting a wide range of protein–protein interactions has been demonstrated. This system has been used to study interactions between known proteins and, perhaps more importantly, to identify new proteins that interact with a known target protein. It has been particularly valuable for studying proteins that control the cell cycle, regulate transcription, and function in oncogenesis and tumor suppression. Given its broad applicability, it seems likely that both this system and other systems that make use of hybrid proteins (see Box 2) will prove useful in many areas of research.

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From initiation to elongation: comparison of transcription by prokaryotic and eukaryotic RNA polymerases

DIRK EICK, ANDREW WEDEL AND HERMANN HEUMANN

Multisubunit RNA polymerases in prokaryotes and eukaryotes share an evolutionarily conserved core. Here, we compare the processes of promoter recognition, transcription initiation and transcript elongation by human RNA polymerase II and by the RNA polymerase of the eubacterium Escherichia coli. Although these two polymerases have diverged widely in structure, important functions have been conserved, suggesting that the basic mechanisms of RNA transcription are similar in eukaryotes and prokaryotes.

RNA polymerases are enzymes that catalyse the formation of 3'–5' phosphodiester bonds between ribonucleoside triphosphates. RNA polymerases can be divided into two evolutionary groups according to their subunit composition. Single-subunit RNA polymerases, such as those found in mitochondria and the bacteriophages SP6 and T7, transcribe a limited number of genes in a small genome. In contrast, those such as RNA polymerase in *Escherichia coli* and RNA polymerase II in eukaryotes are composed of multiple subunits and are responsible for the regulated expression of thousands of genes during cell growth and differentiation. Although RNA polymerases with single and multiple subunits show no obvious sequence homology, the possibility that they have a common evolutionary origin cannot be ruled out[1–3].

Here, we focus on *E. coli* RNA polymerase and human RNA polymerase II. We compare promoter recognition, the formation of closed and open complexes, and abortive and productive transcription, and finally discuss how a recently discovered nuclease activity associated with transcription complexes of both these polymerases might support resumption of transcription when the complex pauses.

Assembly of the transcription complex at the promoter

The RNA polymerase holoenzyme of *E. coli* consists of five subunits: β' (165 kDa), β (155 kDa), two identical α subunits (each 35 kDa), and one of several specific σ initiation factors, the most common of which is σ70 (72 kDa). The spatial arrangement of the components within the holoenzyme has been analysed by electron crystallography and neutron scattering studies[1,4]. The core enzyme, consisting of the subunits β'βαα, forms a triangle, with the two α subunits as the base and the two large subunits β' and β as the sides (Fig. 1a). The σ70 factor binds at the center of this triangle and makes contact with each of the other subunits. σ70 can bind to DNA only when it is bound to the core enzyme. In binding DNA, σ70 makes contact with the promoter around the −35 nucleotide position as well as around the −10 position (the Pribnow box). The core enzyme contains all functions necessary for RNA synthesis, while the σ factor mediates promoter specificity[5].

The holoenzyme protects a DNA sequence of about 50 bp at the promoter and probably makes contact with the DNA via the two elongated large subunits β' and β. The β' subunit has the capacity to bind DNA, while β contains the active site for RNA synthesis. The α subunits appear to be involved in promoter recognition; it has recently been demonstrated for at least some promoters that these subunits make direct contact upstream of the −35 region[6].

RNA polymerase II (pol II) is made up of more than ten subunits. The two largest subunits are clearly related to the β' and β subunits in *E. coli* RNA polymerase. Two subunits with limited homology to the α subunits in the *E. coli* RNA polymerase have also been identified[7,8]. Thus, pol II harbors a core similar to that of *E. coli* RNA polymerase (this is also true for eukaryotic RNA polymerases I and III). The functions of the other subunits of pol II are not clear, and a putative role for these in promoter recognition has not yet been proven.

Technological advances in high-throughput screening

Prabhavathi B Fernandes

A variety of assay technologies continue to be developed for high-throughput screening. These include cell-based assays, surrogate systems using microbial cells such as yeast and bacterial two-hybrid and three-hybrid systems, and systems to measure nucleic acid–protein and receptor–ligand interactions. Modifications have been developed for cell-free, homogeneous assay systems, such as time-resolved fluorescence, fluorescence polarization and the scintillation proximity assay. Innovations in engineering and chemistry have led to delivery systems for nanoliter volumes and sensitive biosensors for ultra-high-throughput screening conducted in nanoliter and picoliter volumes. Spectroscopic methods have been extended to read single molecule fluorescence. Technologies are being developed to identify new targets from genomic information in order to design the next generation of screens

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Abbreviations

FCS	fluorescence cross-correlation spectroscopy
FP	fluorescence polarization
GFP	green fluorescent protein
HTRF	homogenous time-resolved fluorescence
HTS	high-throughput screening

Introduction

The demand for new chemical entities against proven targets, as well as compounds active against new gene products, has made high-throughput screening (HTS) increasingly important as the source of new leads for drug discovery [1••,2••]. HTS is now closely linked to chemical synthesis and the goal is to screen many targets against as many compounds as possible. The large number of leads resulting from increased throughput in screening is expected to result in more compounds entering clinical trials. The quality of these screens is expected to improve the odds of getting new drugs approved. The throughput goals are being met by adapting assays to automation and miniaturization in order to contain reagent costs [3]. Close working relationships between scientists in engineering, photochemistry and other disciplines with HTS groups has resulted in accurate delivery and detection systems. Design and development of sensitive reporters has made microminiaturization of assays possible. HTS is no longer the bottleneck to discovering new leads and an intense effort is underway to identify valid drug targets from new gene sequences to feed the HTS machinery [4]. This review covers recent developments in high-throughput screening technologies and their impact on sensitivity and throughput of screens.

New targets from genomics

Many thousands of new genes are being identified from genome programs. To convert genomic data into useful information for designing screens, however, the functions of the proteins encoded by these new genes must be determined *a priori* [5,6•,7]; therefore, the focus has shifted from genomics to 'proteomics', the science of understanding the function of new proteins. In order to do that, the molecular circuitry or interactions of proteins encoded by the new genes are under investigation. Companies, such as Oxford GlycoSciences (Abingdon, UK), are gathering large-scale information on interacting proteins and identifying those that are specifically expressed in diseased tissue. The differential expression of genes in diseased tissues is an indication that they may be useful drug discovery targets. One approach to develop HTS using the newly identified proteins is to find small molecules that interact directly with the new protein of interest. Gel filtration and mass spectrometry allows the separation and identification of compounds that are bound to the protein [8]. Testing all of the compounds found in these screens, in whole-cell functional assays and animal models is a laborious process. In some instances, even knowing the biological activity of a protein does not allow for the development of a high-throughput screen. For example, knowing the role of leptin in the control of feeding behavior was not sufficient to develop a screen to identify small-molecule agonists. Screening for small-molecule agonists became possible only when the receptor (the *db* gene product) that bound leptin was identified [9]. Small Molecule Therapeutics, Inc (Monmouth Junction, NJ, USA) has a proprietary technology, named FIST (Functional Interactive Screening Technologies), to develop screens for proteins whose biological activities are undefined. Once interacting proteins are found it is possible to set up high-throughput functional screens using a variety of methodologies.

Screen design

Cellular and organism-based systems

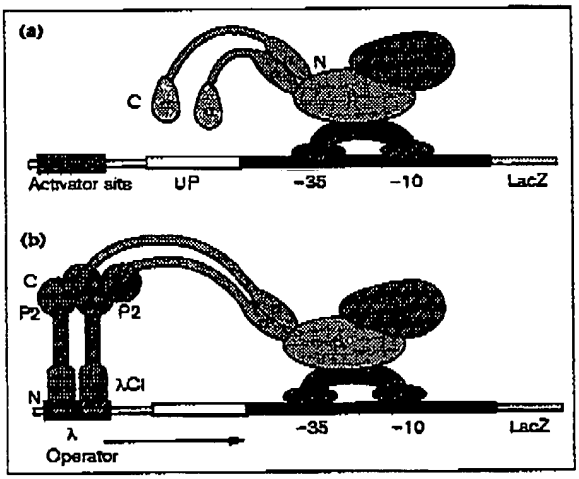
Cell-based assays, using the function of the target protein to obtain a phenotype, mimic the biological role of the protein in a specific disease state more closely than cell-free assays [10]. Fluorescent reporters, such as green fluorescent protein (GFP) and its various mutants, have greatly aided the development of whole-cell assays [11••]. Changes in intracellular calcium levels can be measured using two GFPs linked to calmodulin [12••,13–15]. Changes in the structure of calmodulin brought about by binding calcium brings the GFPs together or drives them apart, resulting in the increase or loss of fluorescence resonance energy transfer

(FRET) between the two GFPs. Mutants of luciferase, another frequently used reporter with long-lived luminescence have been made [16]. Tsein and co-workers [17•] have described another innovative reporter technology using β-lactamase with a fluorescent substrate. In this method, a novel β-lactam substrate is used that fluoresces when the β-lactam ring is cleaved. Whole model organisms, such as *Caenorhabditis elegans*, are also useful for identifying the function of proteins, but are too cumbersome for screening thousands of compounds. Surrogate microbial systems, including yeast and bacterial models, have become a useful alternative to animal and mammalian cell systems for screening [P1,18,19].

Systems for protein–RNA and protein–protein interactions

The nature of the interacting surfaces between proteins and between RNA and protein has made it difficult to find specific small-molecule inhibitors of these interactions. Standard ligand-binding assays have not been particularly useful because they do not take into account the complexity of interactions between multiple facets as well as multiple proteins [20••]. Thus, complex screening systems such as phage display and translation or transcription-based assays have been developed. More recently, an assay based upon translation repression using *Saccharomyces cerevisiae* has been described [21]. In this method, translation is repressed in yeast and mammalian cells when a protein binds specifically to the 5′ end of mRNA. This repression is steric in nature and the physiological function of the protein–RNA interaction is not important. This method, called TRAP (translational repression assay procedure), has been validated with three prototypical systems using yeast: the binding of bacteriophage MS2 coat protein to MS2 replicase mRNA; the binding of the iron regulatory protein 1 to iron-responsive elements; and the binding of spliceosomal protein U1a to loop 2 of U1 small nuclear RNA. TRAP is adaptable to high-throughput screens in yeast and to mammalian cells for the identification of pharmacological agents.

Figure 1



Bacterial two-hybrid protein interaction technology. (a) RNA polymerase holoenzyme is made of 5 subunits, β, β1, 2 αs and σ. It binds to the promoter (−35 to−10). The carboxy-terminal domain interacts with the upstream activator sequence. An easily readable reporter, such as LacZ or β-galactosidase, is read from the promoter. Binding of proteins such as λcI to another upstream activator site stimulates transcription. (b) The carboxy-terminal domain of the α subunits have been replaced with one partner of the interacting protein pair (P1) under study. λcI is a protein with two domains that binds as a dimer to the activator site. When its carboxy-terminal domain interacts with the α subunit of RNA polymerase, transcription is stimulated. The carboxy-terminal domain of λcI is replaced by the second protein (P2) of the interacting protein pair under study. Binding of proteins P1 and P2 brings the RNA polymerase to the λcI activator and transcription of LacZ or β-galactosidase is stimulated. Adapted, with permission, from [28].

The yeast two-hybrid system has been used most frequently to identify interacting pairs of proteins. The use of the yeast two-hybrid system for identifying receptor–ligand interaction was previously described by Kajkowski *et al.* [22] and Pausch [23]. These findings have been extended by Zhu and Kahn [24], who have used a similar system to study the interaction of the extracellular domain of the insulin-like growth factor-1 receptor and proinsulin. A variety of modifications of the yeast two-hybrid system have been developed. A yeast three-hybrid system has also been developed to identify contacts between larger protein complexes such as between the subunits of the RNA polymerase holoenzyme and the transcription/DNA repair factor TFIIH [25]. It has been noted that in a simple two-hybrid configuration, these proteins can be targeted non-specifically by other proteins. The third protein partner in the three-hybrid pair allows for, or prevents, the formation of the transcriptional activator and is regulated by the Met25 promoter. This is easily turned off by adding methionine to the growth medium. A similar three-hybrid system has been used in mammalian cells to identify rapamycin analogs that interact with FK-binding protein (an immunosuppressant) 12 (FKBP12) and FKBP12–rapamycin-associated protein [26]. Proteins binding to these proteins could have the potential for immunosuppressive activity.

The two-hybrid screen, which is widely used with *S. cerevisiae*, is relatively insensitive to pharmaceutical agents because of the nature of its cell wall and membrane, as well as its multidrug efflux systems. Cell permeability by small molecules is less of an issue with the simple bacterial two-hybrid system using *Escherichia coli* as described by Hochschild and co-workers [27••,28]. In this system, the α subunit of RNA polymerase is designed as a chimera, with one protein of an interacting pair fused to the carboxyl terminus of the α subunit. The second protein of the interacting pair is fused to a DNA-bound activator protein, such as the cyclic adenosine monophosphate receptor protein. When these proteins interact, the DNA-bound activator contacts the α subunit, stabilizing the binding of RNA polymerase to the promoter, resulting in transcriptional

activation [28] (Figure 1b). The bacterial two-hybrid system will be more useful in HTS because bacteria can be engineered to be permeable to small-molecule inhibitors. Vectors have been developed for adapting the two-hybrid system to mammalian cells [29]. As a result, multiple strategies are now available for screening for inhibitors of protein–protein interaction.

Microbial systems with increased permeability

In order to overcome multidrug resistance and make yeast more suitable for screening, the function of the ATP-binding cassette (ABC) transporters, such as Pdr5, Snq2 and Yor1, can be disrupted. More recently, it has been noted that the transcription regulators, Pdr1p and Pdr3p, of these efflux pumps down-regulate the expression of hexose transporters Hxt11 and Hxt9 [30]. Overexpression of the hexose transporters coupled to disruption of the ABC transporters yields *S. cerevisiae* with significantly improved drug sensitivity resulting in a useful host organism for screening.

Homogenous systems for screening

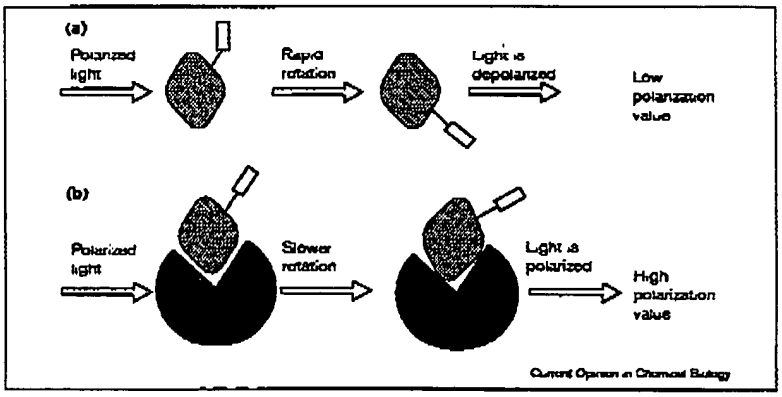
In order to identify small molecules that do not permeate microbial cells, cell-free protein–protein interaction assays and enzyme substrate assays have been developed These use standard ELISA (enzyme-linked immunosorbant assay) formats, homogeneous time-resolved fluorescence (HTRF) and fluorescence polarization (FP) technology [31••]. ELISA assays have not been useful for finding small molecule inhibitors because it is unlikely that small molecules can block high affinity antigen–antibody interactions. Time-resolved fluorometry is being applied increasingly to screening applications [32,33••,34]. Furthermore, this method is adaptable to testing several thousands of samples per day and is based upon FRET. When two fluorescent molecules, a donor and an acceptor, are brought into close proximity to one another, the donor transfers its energy directly to the acceptor. The acceptor, which has different fluorescence properties, emits light at a different wavelength than the donor. This difference is then detected.

In one version of this technology, called homogeneous time-resolved fluorescence (HTRF), the rare earth element lanthanide or europium is protected in the macrocyclic rare earth complex, called cryptate, and is used as the donor. XL-665, a modified allophycocyanin is used as the acceptor. Both of the labels are stable, resist fluorescence quenching and are easily conjugated to biomolecules of interest. The slow fluorescence decay profile of europium can be measured after the initial decay of short-lived background fluorescence seen typically from biological materials. Complexing europium with cryptate protects it from fluorescence quenching and also enhances its fluorescence. Proteins can be directly or indirectly through secondary interactions such as biotin-streptavidin or antibody interaction. As the method is homogeneous and does not require a separation step, it has been applied to enzyme-substrate, receptor-ligand and protein-protein interaction based screening. A time-resolved fluorescence (TRF) method that predates the development of HTRF is readily available from Wallac, AG (Gaithersburg, USA) [33••]. This technology involves the use of lanthanide chelates and europium or terbium as the fluorophore. Initially, Wallac, AG's technology required a separation step after the incubation step, but recent modifications include a number of homogeneous methods.

FP is yet another alternative, nonradioactive, homogenous assay system [35••]. It is based upon the intensity of polarized light emitted from fluorophores in solution when excited with plane-polarized light. The speed at which molecules rotate is dependent upon their size when the viscosity of solvent is constant. When excited with polarized light, smaller molecules emit light that is random or depolarized as they tumble very fast. When small molecules bind other molecules, the retention of polarized light will be proportional to the increase in size and decrease in mobility of the combined molecules (Figure 2). The relationship between polarization of fluorescent molecules

Figure 2

FP to detect changes in size of test molecules. Detection of ligand-receptor binding by changes in FP. (a) A small fluorescent molecule, such as a labeled ligand, rotates rapidly in solution and does not orient in the plane of polarized light. Hence the light is depolarized and the polarization value is low. (b) When small fluorescent molecules or ligands bind to larger macromolecules, such as a specific receptor, the resulting bound molecule does not rotate fast and orients in the plane of polarized light. Hence the light is polarized and the polarization value is higher. Ligand, light gray; fluorescent label, white; receptor, dark grey.



and their rotational diffusion has been exploited extensively in biochemistry and medical diagnostics.

More recently FP has been successfully used for tyrosine kinase assays [36•]. FP can also be applied to receptor–ligand binding, protein–protein interaction, protease, protein–nucleic acid and other interactions where the substrate changes in size after the reaction. The labeling reagents required for FP are less expensive and more readily available than for HTRF or TRF and the labeling procedure is simpler. In some FP applications, large amounts of antibody are required. This drawback has been overcome with the development of a competition immunoassay. In a prototypical competition FP, an unlabeled protein or peptide is used and the reaction product, unlabeled phosphorylated tyrosine peptide, competes with a tracer, labeled-phosphorylated tyrosine peptide, for the antiphosphoryrosine antibody, thus reducing the polarization signal.

Other homogeneous methods, such as scintillation proximity assay have been adapted to HTS by the availability of microplates, where the scintillant is directly incorporated into the plastic of the microtiter wells [37].

Reporter detection technology

Reagent costs for ultra HTS are formidable if sample volumes are in the microliter range; therefore, high density plates have been created for nanoliter volumes. The decrease in reactions volumes has forced the development of very sensitive reporter and reader technologies.

Spectroscopic methods

Fluorescence has traditionally been the most sensitive and efficient method of detection for biological assays. The highest level of sensitivity for fluorescence-based assays is through the use of laser-induced fluorescence detection of single molecules. Burst-integrated fluorescence lifetime (BIFL) spectroscopy is useful for monitoring conformational changes of a single molecule. It combines the advantages of selective TRF spectroscopy and selective fluorescence correlation spectroscopy [38•]. Fluorescence correlation spectroscopy allows quantitative statistical analysis of molecules relative to their kinetic parameters and fluorophore properties. Time resolved fluorescence spectroscopy allows direct measurement and characterization of the molecular state [39••]. Using single molecule spectroscopy it is possible to measure changes that affect a single molecule rather than averaging the changes in populations of molecules in solution. In the example chosen, the conformational changes of a tetramethylrhodamine-labeled DNA duplex in solution were measured using far-field confocal detection coupled to two-dimensional real-time spectroscopy. Thus, fluorescence intensity and lifetime are measured simultaneously on a single molecule. It is likely that BIFL will be used in screening to measure molecules interacting with receptors, DNA and RNA, as well for measuring enzyme function.

Enzymes can generally be classified as performing cleavage reactions or joining reactions. Those that perform cleavage reactions include proteases, nucleases and phosphatases. Those that perform joining reactions include kinases and ligases. Eigen and co-workers [40••,41••] have developed a dual-color fluorescence cross-correlation spectroscopy (FCS) method to measure molecular fragmentation and assembly resulting from the activity of such enzymes. In this method, FCS is coupled to a highly sensitive single photon detector. In the demonstration of the use of this technique, the fluorescence dyes, Cy5 and rhodamine green, were attached to the ends of a double-stranded oligonucleotide in which there was a single EcoR1 restriction enzyme recognition site. The samples were tested at very low concentrations and in femtoliter volumes in order to measure the cleavage of a single DNA molecule. The sampling window was created 'on-the fly' by focusing two lasers at 488 and 633 nm wavelengths into the sample and allowing the path of laser light to cross within the sample volume. The spot at which the two lasers cross is the only part of the sample that is exposed to both wavelengths of laser light. The excited photons that are emitted are collected on dual wavelength detectors. The bifluorescent DNA molecules will emit at the two wave lengths as they diffuse through the intersection of laser light. As the DNA is cleaved by the DNA restriction endonuclease, the correlated fluorescent molecules decrease. Nonenzymatic reactions involving association and disassociation of molecules could also be measured by FCS.

Chemical fluorophores

Chemical sensors or synthetic receptors have been developed for sensing anions in a solution. These synthetic receptors use the principle of photo-induced electron transfer in photosynthesis and have been developed to detect photons [42•,43] In this system, fluorophores are linked to an anion-binding component called the receptor. The interaction of the photon with the receptor changes the fluorescent properties of the fluorophore. An electron is transferred from the receptor to the fluorophore when light is focussed on the receptor. This reduces the excited state of the fluorophore. When the light is turned off, the photon moves back to the receptor and the light turns on. The receptor or the linker can be varied to suit the chemical change that needs to be detected. Different arenedicarboximidide fluorophores have been used to emit different colors to cover the visible and UV light range. In one example, guanidinium is poised as a receptor coupled to a fluorescent probe that can selectively detect anions. The synthetic receptor is made of three guanidinium groups hydrogen-bonded and ion-paired with carboxylate anions in carboxyfluorescein, the fluorophore. The resulting deprotonation makes the receptor absorb ultraviolet light and become highly fluorescent. When an anion such as citrate displaces the carboxyfluorescein, fluorescence is decreased [44]. Lanthanides have been used as the receptor or sensor. When potassium or metal ions bind to the lanthanide, light is emitted when excited with UV light [45]. This technology will add to the fluorescent sensor field

through its use in competition assays. It is being developed further for sensing neurotransmitters.

New technologies not involving the use of fluorophores or labeling and using simple instrumentation are being developed. A liquid crystal 'chip' has been built as a biosensor by sandwiching a liquid crystal, 4-cyano-4'pentylbiphenyl, between a pair of gold films [46•,47]. The liquid crystals are used to amplify and transduce protein binding to the surface for optical readouts. The gold films are rougher in one direction and the anisotropy causes the molecules of liquid crystal to line up in an orderly fashion, which is visualized by polarized light. In the prototype used to demonstrate the use of the liquid crystal as a biosensor, biotin was bound on the surface of the gold film and used to bait avidin. Binding of protein to the gold surface removes the surface anisotropy, disrupting the orientation of the liquid crystal molecules and producing a polarized light image that is nonuniform and colored. This method has potential to become useful for measuring protein–protein, receptor–ligand, ion binding and other biomolecular interactions.

Robotics and automation

The decision to automate multiple tasks is dependent upon the number of assays, the type of sample and the throughput needed. The cost and efficiency of large, core systems capable of multitasking, are compared with smaller, defined automation stations [48•]. Zymark Corporation (Hopkinton, USA) has built a modular system, called the Allegro System, for screening a thousand 96-well plates per 24 hour cycle [49]. The system is built with an eye on conversion to 386-well plates when assays are sufficiently robust to decrease reaction volumes. This capacity for screening is now referred to as ultra-high-throughput. Aurora Biosciences Corporation (La Jolla, USA) has described an integrated ultra-HTS system that is designed to use the fluorescent reporter technologies of β-lactamase and GFP, coupled to its automated screening system [17•,50]. The need to reduce assay volumes even further has led to high density microplates with 1536-wells, 3064-wells and 9600-wells. Piezo-electric ink-jet dispersion is used for sample delivery in these formats. (A Piezo-electric device is excited by voltage to move particles. This technology has been adapted for delivering samples in high-throughput screening.) More recently, Cartesian Technologies (Irvine, California, USA) has developed solenoid ink-jet technology in which a microsolenoid valve is used to generate droplets of five to ten nanoliters. Other methods, such as microcapillary (Genometrix Inc; Woodlands, USA) and solid-pin technologies (Beckman Instruments; Fullerton, USA) are available for transferring small droplets to a nanoplate [51,52••]. These last two methods have the advantage of being suitable for delivering 384 samples that are viscous or particulate simultaneously. Microfluidic chips are being developed by Orchid Biocomputer Inc (Princeton, NJ, USA) for ultra-HTS [53]. Caliper Technologies, Inc (Palo Alto, CA, USA) has developed microfabricated chips with nanoscale fluid handling using electro-osmotic pumping to move the fluid [54]. Microfluidics and chip technology are being combined to develop microreaction vessels to which reagents can be pumped selectively. Future screening is expected to use silicone and glass microchips, with automation re-tooled to high precision robotics.

Conclusions

Screening throughput has been increased to high-throughput and ultra-high-throughput to accommodate the large number of samples being generated from combinatorial chemistry [55]. Thus, the bottleneck for drug discovery is no longer the generation of lead molecules. The challenge for the future is the identification of valid drug discovery targets from the vast amounts of genomic information, as well as the rapid characterization of leads to prove specificity, selectivity and efficacy. Careful selection of valid drug discovery targets and the design efficient screens will move drug discovery to effective HTS.

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Screening of antagonists based on induced dissociation of a calmodulin–melittin interaction entrapped in a sol–gel derived matrix

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Abstract

Sol–gel derived materials offer a unique advantage for the development of sensing and screening platforms in that they allow for the entrapment of multiple species within a confined space. In this work, we show that it is possible to entrap an intact protein–peptide interaction, consisting of bovine calmodulin (bCaM) and melittin, into a sol–gel derived silicate material. Fluorescence emission data demonstrate that the entrapped complex behaves similarly to the complex in solution, and can undergo reversible dissociation upon introduction of the denaturant guanidine hydrochloride. Screening of antagonists of the bCaM–melittin complex was accomplished based on induced dissociation of the entrapped complex, which was followed by measuring the loss of sensitization of Tb(III) luminescence originating from energy transfer from the Trp of melittin to Tb(III) bound in the loops of bCaM. This study shows that entrapped protein–peptide complexes can be used as targets for drug screening or for fluorescence-based biosensing.

Keywords: Protein complex; Calmodulin; Sol–gel; Terbium; Fluorescence

1. Introduction

The development of new methods for screening of antagonists that modulate the state of protein–protein interactions is becoming increasingly important [1–6], particularly given the role of such interactions in the control of cellular processes such as signaling, growth and activity [7,8]. As such, modulation of key protein–protein interactions has been proposed as an important mechanism in the control of human diseases including cancers [2] and viral diseases, such as HIV and herpes [7,8]. This has not only resulted in such complexes becoming the focus of many biophysical studies to elucidate the high resolution structure and ultimately the binding interface of the proteins [9], but has identified protein–protein interactions as extremely important targets for high-throughput drug screening [10,11].

Calmodulin (CaM) is a well known activator and regulator in a number of Ca(II) dependent cellular functions [12]. CaM binds Ca(II) into four binding

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loops, and undergoes a conformational change that exposes critical hydrophobic binding pockets that can accommodate hydrophobic residues on other proteins [13,14]. In this manner, the Ca(II)-activated form of CaM is able to associate with a number of important proteins, such as myosin light chain kinase (MLCK), various other kinases and phosphatases, and several other smaller peptides, including the M13 peptide, melittin (Mel) and mastoparan [4,15–20]. The binding of such species to CaM has been examined using a number of methods, including X-ray crystallography [21,22], nuclear magnetic resonance [23], surface plasmon resonance [4] and fluorescence spectroscopy [20].

A variety of antagonists are known to bind to the hydrophobic domains of Ca(II)-activated CaM. Typical antagonists are derived from phenothiazine species, and include trifluoperazine, chlorpromazine, fluphenazine, and acetopromazine [24]. Another potent inhibitor is calmidazolium (compound R24571). Binding of such species by Ca(II)-activated CaM inhibits the binding of specific binding partners such as MLCK or melittin. These compounds can also dissociate intact CaM–protein complexes, leading to their potential use as drugs [24].

In order to develop high-throughput screening (HTS) methodologies, it is necessary to be able to run a large number of assays simultaneously using a limited amount of reagents per assay. Furthermore, it is desirable to use assay formats that involve reversible dissociation of protein–protein interactions, allowing for the potential to reuse expensive protein-based reagents. To achieve these goals, it is useful to immobilize proteins, in their native conformations, onto solid supports. The immobilization protocol can be used to retain protein–protein interactions in a localized region, and if incorporated with a size-exclusion matrix, may allow passage of low molecular weight drug candidates into the matrix while preventing leaching of protein partners. Such an immobilization scheme could also lead to long-term advancements in HTS based on affinity chromatography [25] or protein microarray technologies [10].

An emerging method for immobilization of proteins is their entrapment into a porous, inorganic silicate matrix that is formed via a low-temperature sol–gel process [26]. Numerous reports have appeared describing both fundamental aspects of entrapped proteins, such as their function [27–37], structure [38–40], dynamics [41–43], accessibility [40,44], reaction kinetics [38,45] and stability [46–53], and their many applications for catalysis, sensing, and affinity chromatography [54]. However, no reports yet exist describing the use of sol–gel entrapped proteins or protein–peptide interactions for drug screening.

In this work, we have examined the behavior of a model protein–peptide interaction involving bovine calmodulin (bCaM) and melittin when entrapped in a sol–gel derived glass, and the potential of the immobilized complex to respond to drug candidates. Melittin, which is an active component of bee venom, is a cationic peptide composed of 26 amino acids with a molecular weight of 2840 [55]. The peptide contains a single tryptophan (Trp) residue which undergoes a significant change in emission properties upon binding of the peptide to CaM [56], making it amenable to fluorescence-based studies of the CaM–Mel interaction. The presence of the Trp residue within melittin also allows for a homogeneous time-resolved fluorescence assay of the bCaM–Mel interaction based on sensitization of long-lived Tb(III) luminescence from Tb(III)-loaded CaM by the Trp residue of the peptide. Herein, we report on the effect of entrapment on the complex between bCaM and melittin using both the intrinsic tryptophan (Trp) fluorescence signal of the peptide and sensitized Tb(III) luminescence. We show that it is possible to entrap the intact protein–peptide interaction into a sol–gel derived material, that the dissociation of the interaction can be distinguished spectroscopically, and that the entrapped complex can be modulated using known antagonists, indicating that the entrapped protein–peptide interaction can be used for sensing or screening of antagonists.

2. Experimental

2.1. Chemicals

Tetraethyl orthosilicate (TEOS, +99.999%), acrylamide (99%), terbium(III) chloride (hexahydrate, 99.9%), benzamadine, trifluoperazine (TFP), acetopromazine, chlorpromazine, fluphenazine, calmidazolium (compound R24571), and dipicolinic acid (DPA, 99%) were supplied by Aldrich. Phosphodi-

esterase 3′,5′-cyclic nucleotide activator from bovine brain (calmodulin, bCaM, ~95%), melittin (Mel, ~85%) and polymethacrylate fluorimeter cuvettes were purchased from Sigma. Anhydrous calcium chloride (99.9%) was purchased from Fisher Scientific (Toronto, ON). Guanidine hydrochloride (GdHCl, Sequanol grade) was purchased from Pierce (Rockford, IL). All water was purified by reverse osmosis and de-ionized through a Milli-Q four stage water purification system. All other chemicals were of analytical grade and were used as received.

2.2. Procedures

2.2.1. Preparation of bioglasses

TEOS was hydrolyzed as described previously [38]. Equal volumes of hydrolyzed TEOS and a Ca(II)-loaded PIPES buffer (10 mM PIPES, 100 mM KCl, pH 6.5) containing 7.5 μM of bCaM and 7.5 μM of melittin (1:1 molar ratio unless otherwise stated) were mixed, providing a total volume of 600 μl (2.25 nmol of each partner in cuvettes) or 200 μl (0.75 nmol of each partner in 96-well plates). The plates and cuvettes were sealed with Parafilm™, and cuvettes were placed on their side and allowed to stand at room temperature until gelation occurred. A small hole was punched in the Parafilm™ to allow solvent to escape, and the resulting materials were aged in air at 4 °C for 15 days before fluorescence studies were done to prevent leaching of melittin. During this time the material shrank in volume by a factor of ~2 owing to loss of internal solvent. Partially dried glasses aged in this manner have previously been shown to have average pore diameters of ca. 6–8 nm [57], with the pore volume being ~50% of the total volume of the glass [26]. These pore sizes should be sufficient to allow dissociation of the bCaM–Mel complex (X-ray crystallography indicates that the complex is relatively compact, being approximately 2 nm in diameter [58]). Based on these values, one can roughly estimate that the concentration of the bCaM and Mel in the glass are approximately double the initial concentration (i.e. 300 μl of buffer are added to form a 600 μl glass, which shrinks to ~300 μl total volume and has ~150 μl of internal solvent). However, given the inaccurate nature of this estimate, all drug:protein ratios are given as mole ratios, since these are independent of volume.

2.2.2. Characterization of entrapped bCaM–Mel interaction

Steady-state fluorescence measurements of samples in cuvettes were performed using a SLM 8100 spectrofluorimeter (Spectronic Instruments, Rochester, NY), as described elsewhere [38]. Trp emission spectra from the peptide were excited at 295 nm with emission collected from 305 to 450 nm when no Tb(III) was present, or 305–560 nm for experiments involving Tb(III). All spectra were collected using 4 nm bandpasses on both the excitation and emission monochromators to avoid direct sensitization of Tb(III) by Tyr-99 in binding loop III of bCaM (under these conditions the directly sensitized signal was < 4% of the signal obtained from the bCaM–Mel complex). Assays involving time-gated detection of Tb(III) luminescence were done in 96-well plates using a Spectramax Gemini fluorescence platereader (Molecular Devices, Sunnyvale, CA). Excitation of Trp was done at 297 nm, with Tb(III) luminescence monitored at 545 nm using a 250 μs time delay between excitation and detection of the emission. This instrument has fixed bandpasses of 9 nm on the excitation and emission monochromators, and in this case, the directly sensitized Tb(III) signal was 20% of the total Tb(III) signal obtained from the bCaM–Mel complex. All Tb(III) emission data obtained using the platereader were corrected for this background contribution by using a Tb(III)-loaded bCaM sample as a blank. The state of the complex was further assessed by fluorescence quenching studies. Free and entrapped complexes were titrated with aliquots of 8.0 M acrylamide dissolved in PIPES buffer containing a metal-ion concentration identical to that in the sample buffer. Integrated emission intensities and/or intensity-weighted mean lifetimes were determined at various quencher concentrations, and the data was analyzed using the Stern–Volmer equation [59].

Time-resolved fluorescence intensity decay data of the Trp residue of melittin was acquired in the time-domain using a PTI laserstrobe fluorimeter (Photon Technologies Inc., London, ON), as described elsewhere [60]. Samples were excited at 295 nm and the emission intensity data was collected at 340 nm using 4 nm bandpasses under magic angle conditions into 25 ps time windows, starting 2 ns before the laser pulse arrived and covering a 25 ns range. The instrument response function was collected by measuring

the Rayleigh scattering of the laser pulse from water, and was used to deconvolute the instrument response from the experimentally determined decay trace. Appropriate baseline offset and time-shift parameters were obtained by allowing these to be floating parameters in the fit. The decay was fit to a discrete decay model using methods that are described elsewhere [60].

2.2.3. Dissociation studies

Dissociation of the complex was attempted using pH, urea, GdHCl and temperature changes, and indicated that reversible dissociation was possible using GdHCl. To dissociate the intact complex, the protein–peptide interaction was incubated in 2 ml of a solution of 2.0 M GdHCl for 20 min. Removal of the denaturant by dialysis (for the free protein) or by rinsing (for the entrapped protein) was done until the fluorescence emission spectra showed no shifts upon further dialysis or rinsing. Dialysis of species in solution was typically accomplished using five exchanges of 100 ml of fresh buffer over 24 h. For entrapped species full recovery was accomplished using five rinses of 2 ml of fresh buffer with a 5 min equilibration time. Steady-state and time-resolved fluorescence was measured for the intact, dissociated and recovered complexes to determine the extent of dissociation and recovery in solution and when entrapped. The state of the complex was also assessed by monitoring the recovery of Tb(III) luminescence from a solution containing a molar level of 4:1 Tb:bCaM.

2.2.4. Drug interaction studies

All drug interaction studies were done using a 1:1 bCaM:Mel complex that had Tb(III) present at a 4:1 molar ratio of Tb(III):bCaM. The drug compounds were added at mole ratios ranging from 2:1 to 20:1 (drug:bCaM) to the intact complex that was either in solution or entrapped into TEOS derived glasses present in the wells of a 96-well platereader and incubated for 24 h to ensure that equilibrium was reached. The changes in the Tb(III) emission were monitored and corrected for direct Tyr–Tb(III) sensitization and for any quenching effects observed from interactions of the drugs with the Trp residue of melittin. Estimated inhibition constants (K_I) were obtained by first determining the total change in corrected fluorescence intensity between F_{init} (initial intensity) and F_{final} (the fluorescence intensity at the point where the intensity change leveled out). The drug concentration at which the fluorescence intensity had changed by one-half of its final value was assigned as the K_I value, assuming 1:1 binding of the drug and the complex.

3. Results and discussion

3.1. Solution-based studies

The fluorescence properties of the bCaM–Mel complex were first assessed in solution to provide a basis for comparison to studies involving the entrapped complex. Table 1 shows the steady-state and time-resolved fluorescence and quenching data for free melittin (with and without GdHCl) and for the intact, dissociated and recovered complex of holo (Ca(II)-loaded) bCaM and melittin. On the formation of a complex by addition of bCaM to the peptide, the Trp emission intensity of melittin increased by approximately 30% and the maximum emission

Table 1
Fluorescence properties of intact, dissociated and re-associated calmodulin–melittin in solution

Solution	Relative intensity (±0.05)	τ ± 0.3 ns	λ (nm) ± 1 nm	k_q ($M^{-1}\,s^{-1}$) ± 0.2
Native	1.00[a]	3.39	335	0.8×10^9
Denatured	0.57	2.35	345	4.1×10^9
Recovered	0.91	3.26	335	0.5×10^9
Melittin	0.77	4.28	345	5.0×10^9
Melittin (GdHCl)	0.92	2.10	345	4.5×10^9
Melittin (recovered)[b]	0.40	2.30	345	5.0×10^9

[a] All intensity values are relative to the intensity obtained for the native bCaM–Mel complex.
[b] Recovery of melittin by dialysis led to significant aggregation of the peptide, and also led to some leaching of the peptide, which lowered the final intensity value.

wavelength of the Trp residue blue-shifted by ca. 12 nm. These changes reached a plateau at a protein–peptide ratio of 1:1 and are indicative of the formation of an intact complex [61,62]. The formation of the complex also led to a decrease in the mean lifetime of Trp emission in melittin (by ca. 1 ns) and a decrease in the k_q value by six-fold, indicative of a more solvent-shielded Trp residue. The addition of Tb(III) to the holo-bCaM–Mel complex at a 4:1 molar ratio (to fill all the CaM binding loops) resulted in intense Tb(III) luminescence when excited at 295 nm [63] (described in more detail later), while essentially no Tb(III) luminescence was detected for the peptide alone or in the presence of bCaM alone when excitation was done at 295 nm using 4 nm bandpasses. As indicated above, use of 9 nm bandpasses (when using the platereader) led to direct Tyr–Tb(III) sensitization that produced a Tb(III) emission intensity that was ~20% that of the intact complex. This value was subtracted from Tb(III) signals that were obtained from the bCaM–Mel complex to produce corrected fluorescence values. Identical fluorescence studies carried out with apo calmodulin (formed using an excess of EGTA) resulted in no significant changes in fluorescence properties upon addition of melittin, indicating that the apo-bCaM and melittin were unable to interact and form a complex, consistent with previous reports [64].

Addition of 2.0 M GdHCl to the intact complex produced a shift in the fluorescence properties, with values comparable to the free peptide being observed for the emission wavelength and bimolecular quenching rate constant. The intensity and lifetime values also shifted, however, they were not similar to those of the free peptide, but rather corresponded to the values obtained for melittin in 2.0 M GdHCl (see Table 1), indicating that the GdHCl must induce a structural change in the peptide that alters the Trp emission (this may be tetramer–monomer dissociation, which is known to occur for melittin) [65]. The similarity in the fluorescence parameters for the complex and the peptide in 2.0 M GdHCl indicated that this level of denaturant was sufficient to dissociate the complex, although it is not clear whether some residual interaction may have remained between the binding partners (see later). This concentration of denaturant also reduced the Tb(III) signal to a value close to that of the peptide alone (<10% of full signal), although the signal was not completely eliminated, even at much higher GdHCl levels (8.0 M), possibly due to Tb(III) induced aggregation of the peptide [66] or incomplete dissociation of the complex.

Removal of the denaturant by dialysis resulted in a full recovery of the wavelength, mean lifetime and bimolecular quenching rate constant of the Trp residue to values that were within error of those of the original complex. However, it was not possible to fully recover the intensity value for either the Trp or Tb(III) peaks, likely due to dilution encountered during the dialysis stage of the procedure and/or leaching of a small fraction of the peptide from the dialysis bag. Similar problems were encountered when attempting to recover the fluorescence properties of the peptide upon dialysis of GdHCl. The results, shown in Table 1, suggest that the peptide likely leached from the dialysis bag and may have also underwent a conformational change leading to a lower emission lifetime. Despite these problems, the data clearly shows that several fluorescence parameters are able to accurately follow the state of the complex, indicating that such signals should be able to report on the state of the entrapped complex.

3.2. Characterization of entrapped complexes

Fig. 1 shows the emission spectra of the native and denatured complex in solution as compared to

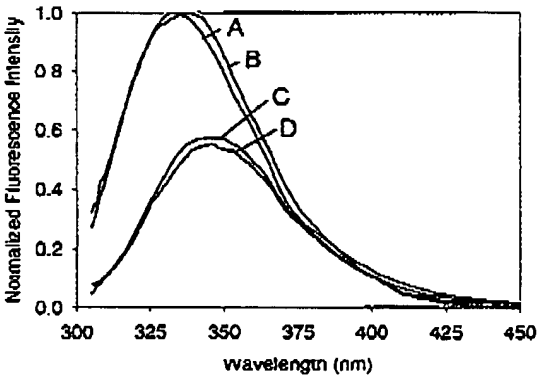

Fig. 1. Steady-state fluorescence emission spectra of free and entrapped bCaM–melittin complex in the native and denatured states. (A) native complex in solution, (B) native complex entrapped in sol–gel derived silica, (C) denatured complex in solution, (D) denatured complex entrapped in sol–gel derived silica

the entrapped complex in the native and denatured states, clearly indicating that entrapment does not significantly alter the spectral properties of either state. In each case, the expected blue shift upon binding of melittin to bCaM occurs, consistent with the interaction of melittin with the hydrophobic binding pocket of CaM, which causes the Trp to move into a hydrophobic region. This result suggests that the complex remains intact during entrapment and is not dissociated by the ethanol that is present during the early stages of the glass formation [67]. The entrapment of either melittin or the apo form of calmodulin with melittin resulted in emission spectra that were consistent with the free peptide (i.e. no formation of a complex), as was found in solution. Fluorescence intensity, lifetime and emission wavelength for melittin within glasses formed with varying ratios of bCaM:melittin (0:1–2:1) indicated that complete formation of the complex was obtained at a molar ratio of 1:1 (results not shown), suggesting that the dissociation constant for the complex was not affected by entrapment in silica.

Table 2 shows the steady-state and time-resolved fluorescence and quenching data for the entrapped peptide and the intact complex together with data for the denaturation and recovery of these species after 15 days of aging. In comparing the emission properties of the entrapped complex (native, denatured and recovered) to those of the complex in solution, it is apparent that the relative changes in fluorescence properties for free and entrapped species are similar in each case (within experimental error). These results indicate that fluorescence can be used to follow the state of the entrapped complex, and confirm that entrapment does not restrict the ability of the complex to undergo dissociation and reformation. This result also suggests that the entrapped complex may be able to be reused multiple times for screening or sensing purposes, resulting in a significant cost savings relative to the use of proteins in solution.

The fluorescence properties of entrapped melittin (in the absence of bCaM) were also examined (Table 2), but in this case the fluorescence data for the entrapped species was often quite different than the values obtained in solution. For example, it was found that the emission intensity of the Trp residue of entrapped melittin in the presence of GdHCl was significantly lower than in solution, suggesting that the presence of silanol groups within the glass may have partially quenched the Trp fluorescence [68] (note that the intensity of recovered melittin in solution is anomalously low owing to leaching of the peptide during dialysis). Furthermore, the bimolecular quenching rate constant (k_q) values obtained for the entrapped peptide were typically much lower than the corresponding solution values, suggesting that the peptide may have associated with the silica surface, thereby hindering the access of the acrylamide to the tryptophan residue. Overall, the results suggest that the presence of bCaM results in almost complete alleviation of electrostatically-induced adsorption of the peptide on the silica surface (as demonstrated by the better recovery of the entrapped complex relative to the entrapped peptide), suggesting that the denaturant does not fully dissociate the complex.

To further assess the state of the entrapped complex and move toward an assay method that was amenable to high-throughput screening, Tb(III) was added to the complex to derive a long-lifetime energy transfer system involving the Trp residue of melittin as the donor, and Tb(III) within CaM as the acceptor. In this system, intense Tb(III) luminescence should be observed for the intact complex, but not for the denatured complex

Table 2
Fluorescence properties of intact, dissociated and re-associated calmodulin–melittin entrapped in a sol–gel derived glass matrix

Entrapped	Relative intensity (±0.05)	$\tau \pm 0.3$ ns	λ (nm) ± 1 nm	k_q ($M^{-1} s^{-1}$) ± 0.2
Native	1.00[a]	2.85	333	0.5×10^9
Denatured	0.55	2.55	344	4.5×10^9
Recovered	1.08	3.01	333	0.3×10^9
Melittin	0.75	4.51	344	3.3×10^9
Melittin with GdHCl	0.64	2.15	344	2.8×10^9
Melittin (recovered)	0.70	4.10	344	3.9×10^9

[a] All intensity values are relative to the intensity obtained for the native bCaM–Mel complex.

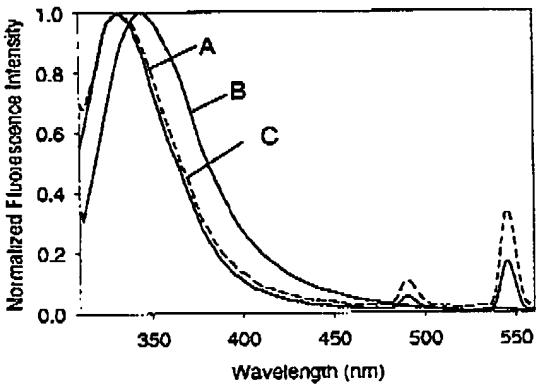

Fig. 2. Emission spectra of Tb(III)-loaded bCaM–Mel complexes entrapped in a sol–gel derived glass (A) native complex (solid line), (B) dissociated complex (broken line); (C) reformed complex (solid line). Note that the spectrum of melittin alone was similar to the dissociated complex, and is not shown.

or for melittin alone. The data shown in Fig. 2 confirms that a terbium signal could be seen for the intact complex but not for the denatured complex or for melittin alone, again indicating that the entrapped complex remained intact. The data demonstrate that the GdHCl is able to enter the glass and at least partially dissociate the complex, resulting in a large change in the Tb(III) signal. It is worth noting that the Tb(III) signal recovers to a value that is somewhat higher that the value obtained for the native protein (Fig. 2). Subsequent denaturation/recovery cycles consistently resulting in Tb(III) signals that were similar to that obtained after the initial recovery cycle (data not shown). The origin of the initial Tb(III) intensity increase during the first denaturation/recovery cycle is unknown, but may be due to a different conformation for the recovered complex relative to the native complex.

3.3. Screening of antagonists using the entrapped complex

Having established that the entrapment of a viable protein–peptide complex within a sol–gel derived glass was possible, our next goal was to demonstrate that such a complex could be used to detect species that are known to alter the state of the complex. We therefore investigated the ability of the entrapped complex to respond to several known calmodulin antagonists, including calmidazolium (compound R24571, IC_{50} = 0.06–0.5 μM) [69,70], fluphenazine (IC_{50} = 11–19 μM) [69,71], trifluoperazine (IC_{50} = 8–52 μM) [4,69], chlorpromazine (IC_{50} = 18–28 μM) [69] and aceiopromazine (IC_{50} > 181 μM) [72], as well as Benzamadine, which acted as a negative control.

Fig. 3 shows the response of the Tb(III) luminescence signals upon addition of the drugs to the intact complex in solution (panel A) and when entrapped into TEOS derived materials (panel B), respectively. These results are corrected for direct sensitization of Tb(III) luminescence by bCaM and for direct quenching of the Tb(III) luminescence by the drugs (which was typically <10%). The data show that compound R24571, fluphenazine, trifluoperazine, chlorpromazine and acetopromazine each resulted in significant changes in Tb(III) luminescence for both the free and entrapped complex. Furthermore, the negative control compound benzamadine resulted in no changes in Tb(III) luminescence (within error). An important point to note is that the initial Tb(III) luminescence intensity obtained for entrapped proteins is often significantly higher than in solution (particularly at low drug concentrations). The origin of this phenomenon is not fully understood, and is currently under investigation.

Table 3 shows the inhibition constants obtained for the drugs using the Tb(III)-based assay in solution and for the entrapped complex, in comparison to literature values. The data clearly showed that the estimated K_I values were in reasonable agreement with the literature values, indicating that the new screening method was able to accurately report drug-induced dissociation of the protein–peptide complex. It is worth noting that K_I values estimated for the entrapped complex were generally within error of the values obtained in solution, showing that the entrapped species were accessible to the drugs and were able to undergo drug-induced conformational changes.

It should be noted that addition of many of the drugs to the complex resulted in an extremely large fluorescence peak originating from the drug, the magnitude of which often obscured the Trp emission spectrum. As a result, it was not possible to use steady-state Trp intensity or emission wavelength values as signals to follow the state of the complex. However, Tb(III) luminescence, monitored in a time-resolved fashion (250 μs delay), was able to generate an intensity-based optical

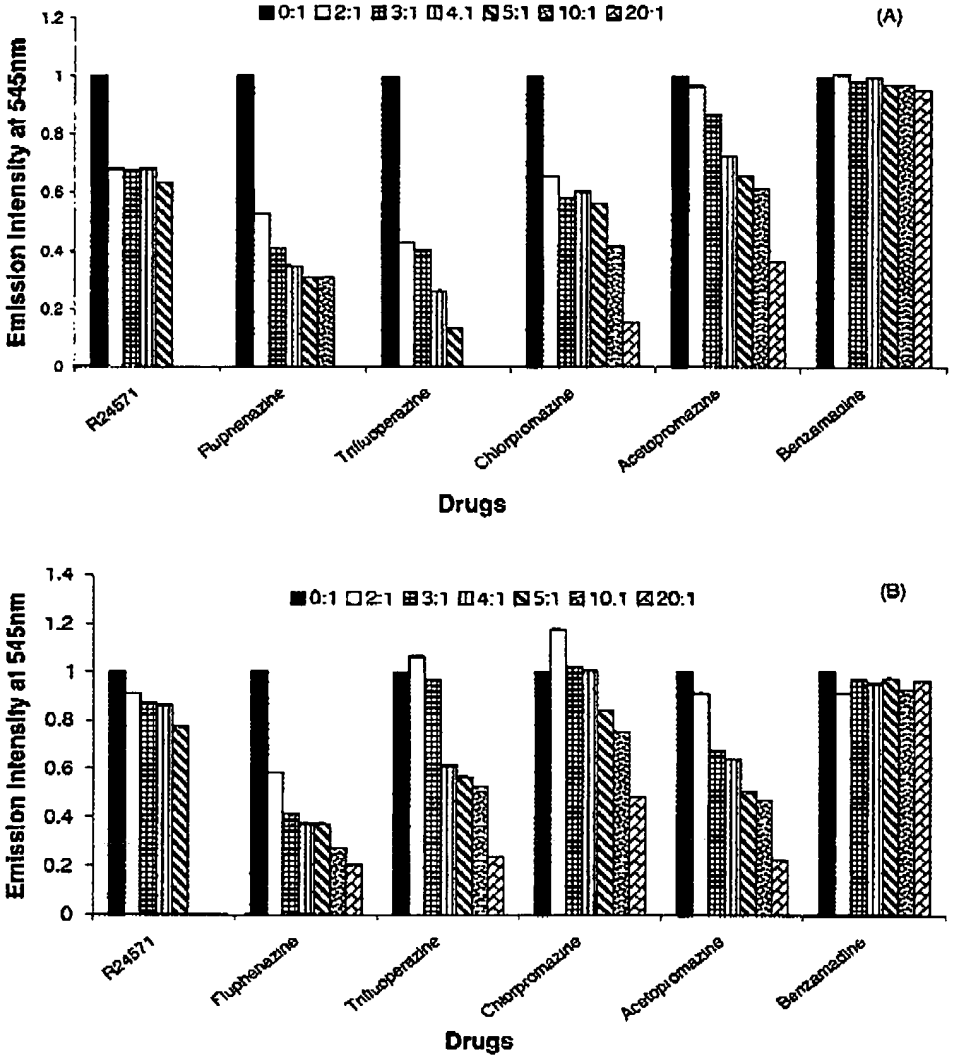

Fig. 3. Relative Tb(III) luminescence intensity for bCaM–Mel in the presence of varying levels of antagonists. The mole ratios of drug:bCaM are shown in the figures. Note: the mole ratios of R24571 are 0:1, 0.025:1, 0.05:1, 0.1:1 and 0.15:1. Luminescence values are shown for bCaM–Mel (A) in solution and (B) entrapped in TEOS derived silica.

signal that could follow the state of the complex with no interference from drug fluorescence. Removal of drugs by dialysis resulted in full recovery of the Tb(III) signal for the entrapped species, confirming that the complex reassociated when the drug was removed, and thus could be reused. The results presented above also suggest that entrapped protein–protein complexes may be able to be applied as biorecognition elements for sensors. Such compounds could be potential mutagens and/or cellular pathway inhibitors, and as such would benefit from the development of sensing platforms based on protein–protein interactions.

Table 3
Inhibition constants obtained for antagonist induced dissociation of the calmodulin–melittin complex in solution and when entrapped in a sol–gel derived glass

Compound	Literature IC_{50} (μM)	IC_{50} (solution, μM)	IC_{50} (entrapped, μM)
Calmodulin (R24571)	0.06–0.5 [69,70]	<0.5	<0.5
Fluphenazine	11–19 [69,71]	11 ± 2	14 ± 5
Trifluoperazine	8–52 [4,69]	12 ± 3	25 ± 6
Chlorpromazine	18–56 [69,72]	28 ± 12	36 ± 9
Acetopromazine	>181 [72]	70 ± 25	70 ± 25
Benzamadine (control)	NB	NB	NB

NB: no binding.

4. Conclusions

We have demonstrated that it is possible to entrap an intact protein–peptide interaction into a sol–gel derived material, and that such complexes can be dissociated by antagonists with K_I values similar to those obtained in solution, and reformed by simply rinsing the drug away. Fluorescence spectroscopy was shown to be able to follow the state of association of the complex, demonstrating the advantages of transparent silica based glasses for spectroscopic studies. We also show that it is possible to utilize energy transfer from the Trp of the peptide to Tb(III) that is bound by calmodulin, producing a very sensitive means of assessing the state of the complex. Luminescence decay of lanthanides generally occurs on the millisecond time scale, making it possible to develop a sensitive assay for assessing the state of the complex using time-gated detection to remove background interferences, as demonstrated herein. The ability of small molecule inhibitors to enter the glass and reversibly dissociate the complex indicated that the entrapped complex is suitable for screening of antagonists in a high-throughput format.

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